# UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Microbiología III



## TESIS DOCTORAL

# Desarrollo de métodos moleculares para la detección y tipificación de cepas de levaduras con interés industrial

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR


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Directora

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# “DESARROLLO DE MÉTODOS MOLECULARES PARA LA DETECCIÓN Y TIPIFICACIÓN DE CEPAS DE LEVADURAS CON INTERÉS INDUSTRIAL”

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# "DESARROLLO DE MÉTODOS MOLECULARES PARA LA DETECCIÓN Y TIPIFICACIÓN DE CEPAS DE LEVADURAS CON INTERÉS INDUSTRIAL"

Tesis doctoral presentada por Petra Wrent para optar al grado de Doctor en Biología por la Universidad Complutense de Madrid

**Directora:**

Dra. María Isabel de Silóniz Jiménez

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**I rörelse**

Den mätta dagen, den är aldrig störst.
Den bästa dagen är en dag av törst.

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd.

Det bästa målet är en nattlång rast,
där elden tänds och brödet bryts i hast.

På ställen, där man sover blott en gång,
blir sömnen trygg och drömmen full av sång.

Bryt upp, bryt upp! Den nya dagen gryr.
Oändligt är vårt stora äventy

***Karin Boye (1900 -1941), escritora sueca***

**In motion**

The sated day is never first.
The best day is a day of thirst.

Yes, there is goal and meaning in our path
but it's the way that is the labour's worth.

The best goal is a night-long rest,
fire lit, and bread broken in haste.

In places where one sleeps but once,
sleep is secure, dreams full of songs.

Strike camp, strike camp! The new day shows its light.
Our great adventure has no end in sight

***Traducido por David McDuff 2005***

Dedicated to

my family

# Resumen

# "Desarrollo de métodos moleculares para la detección y tipificación de cepas de levaduras con interés industrial"

## Introducción y objetivos

El objetivo general e hilo conductor de esta Tesis es el desarrollo de métodos moleculares para la detección y tipificación de cepas de levadura que sean rápidos, económicos y, sencillos de aplicar en la industria alimentaria. Además, como demostración de la utilidad de los métodos desarrollados, se analiza su aplicación a un caso de deterioro de yogures ecológicos.

Las especies en las que se centra este trabajo son interesantes desde varios puntos de vista (deterioro de alimentos, cultivos iniciadores, agentes de biocontrol y ámbito sanitario). El género *Zygosaccharomyces* agrupa especies osmotolerantes, altamente fermentativas y capaces de resistir conservantes como el ácido benzoico y el sórbico. Por ello, este género se considera como uno de los más peligrosos dentro de las levaduras deteriorantes de alimentos. A pesar de este potencial deteriorante, la especie *Z. rouxii*, junto con algunas especies de hongos filamentosos, se utiliza en la fermentación temprana de alimentos orientales ya que mejora sus propiedades organolépticas. En estos ambientes hostiles, por ejemplo en la industria del *miso*, se han detectado cepas híbridas mucho mejor adaptadas; incluso se han generado artificialmente. Por ello, consideramos que la detección diferencial de las cepas híbridas es fundamental y de gran interés. Aunque algunos autores habían descrito métodos para la tipificación de cepas, especialmente para la especie *Zygosaccharomyces rouxii,* hasta algunos de los resultados incluidos en esta Tesis, ninguno de ellos era discriminatorio por sí mismo.

La especie *Debaryomyces hansenii* se encuentra distribuida ampliamente en la naturaleza y, es muy relevante por su halotolerancia y sus aplicaciones industriales, entre ellas el proceso de madurado de los quesos tradicionales y de productos cárnicos como el jamón o los embutidos. Algunas cepas, que previamente se incluían dentro de esta especie, actualmente se clasifican dentro de las nuevas especies *D. fabryi, y D. subglobosus*. Dado que fisiológicamente las tres especies son muy parecidas, se han producido errores al identificarlas. Por otro lado, la identificación mediante el análisis de secuencias, aunque precisa, no siempre es adecuada por su precio para la práctica industrial a gran escala. También en este caso, aunque se habían propuesto algunas técnicas moleculares como alternativa a la identificación tradicional de esta especie, en esta Tesis se demuestra que dichas técnicas no son exactas o suficientemente asequibles.

Con respecto a *Meyerozyma,* es un nuevo género que incluye las especies anteriormente denominadas *Pichia caribbica* y *P. guilliermondii,* ahora *Meyerozyma caribbica* (anamorfo, *Candida fermentati)* y *M. guilliermondii* (anamorfo, *C. guilliermondii*). *M. guilliermondii* se encuentra ampliamente distribuida y suele aislarse de frutas y otros alimentos. Además, figura entre las 17 levaduras ascomicetas más frecuentemente implicadas en infecciones. Se han publicado algunos trabajos con diferentes abordajes, como la diferenciación de cepas con distintos grados de producción de 4-etilfenol, un producto deteriorante de vino o que permiten distinguir las cepas aisladas de muestras clínicas de las que no lo son, con un resultado desigual. Algunos autores demostraron que en realidad no discriminaban entre cepas sino entre las especies *M. guilliermondii* y *M. caribbica.* Por lo tanto, a pesar de la amplitud de su área de interés, aun no existía un

método fiable de tipificación de cepas hasta algunos de los resultados recogidos en esta Tesis.

Los **objetivos** específicos de esta Tesis fueron:

1. Desarrollar un método de tipificación que por sí solo permita la diferenciación de cepas en diferentes especies del género *Zygosaccharomyces*.
2. Esclarecer el posible origen híbrido de las cepas de *Z. rouxii* CECT 11923 y CECT 10425.
3. Desarrollar un método molecular para la detección de cepas híbridas de *Zygosaccharomyces.*
4. Desarrollar un método rápido y económico para la detección específica de la especie *D. hansenii*.
5. Desarrollar un método para la tipificación intraespecífica de la especie *M. guilliermondii.*
6. Estudiar y analizar la aplicación de alguno de estos métodos en un caso de deterioro de yogures ecológicos causado por *M. guilliermondii*.

**Resultados y Discusión**

Con respecto al género *Zygosaccharomyces* y con el fin de desarrollar un protocolo de tipificación de cepas, en este trabajo hemos analizado el método IGS-PCR RFLP del rDNA. El tamaño de los fragmentos amplificados difirió en siete de las nueve especies estudiadas. Dentro del género *Zygosaccharomyces* el tamaño oscilaba entre 4200 pb (*Z. mellis* y *Z. rouxii*) y 7000 pb (*Z. kombuchaensis*). La amplificación de tamaños tan grandes fue posible gracias a la introducción de modificaciones en el método previamente descrito en nuestro laboratorio. Aunque ninguna de las endonucleasas (*Hap*II, *Hha*I y *Mbo*I) son útiles para la discriminación de cepas utilizadas de forma independiente, la combinación de los tres patrones obtenidos permite diferenciar las cepas en un 100% en Z. *rouxii* y Z. *mellis* y un 70% en *Z. bailii*. Es de destacar que todas las cepas de *Z. rouxii* presentan un conjunto de bandas comunes. El análisis de los fragmentos de restricción nos permitió detectar dos cepas de *Z. mellis* que nosotros consideramos mal identificadas (CBS 711 y CBS 7412), las cuales presentaban las bandas características de las cepas de *Z. rouxii*. La secuenciación del dominio D1/D2 del gen 26 S del rDNA y de la región 5,8-ITS del rDNA nos permitió confirmar que esas cepas pertenecen a la especie *Z. rouxii*. A diferencia de otros métodos publicados con anterioridad, que necesitan la combinación de varias técnicas, el método que proponemos es suficiente por si mismo para la tipificación de las cepas.

La aplicación del método mencionado más arriba agrupa a las cepas de *Z. rouxii* CECT 11923 y 10425 en un *cluster* separado junto con las cepas híbridas de *Zygosaccharomyces* (NCYC 1682, NCYC 3060 y NCYC 1682). Por

ello, en este trabajo tratamos de dilucidar la naturaleza de las mismas. Hemos podido comprobar que el tamaño de la región IGS1-rDNA variaba entre cepas (de 1300 pb a 1600 pb) y presentaba un gran polimorfismo tanto entre las diferentes cepas como entre diferentes clones dentro de la misma cepa. Con el fin de evitar los errores de identificación debidos a las especies parentales y teniendo en cuenta el papel relevante de los híbridos, cada vez más reconocido, en este trabajo hemos diseñado unos cebadores específicos (HibZF/HibZR) que reconocen secuencias específicas que están presentes en la región IGS1-rDNA de las cepas híbridas (incluidas las especies CECT11923 y CECT10425) pero no de las especies que no lo son, ya sean de *Z. rouxii* como de otras especies ensayadas. Basándonos en los resultados obtenidos con los cebadores específicos de especie, la secuencia del dominio D1/D2 y de las copias divergentes de la región 5,8S ITS, concluimos que la cepa CECT 11923 es un híbrido entre *Z. rouxii* y *Z. pseudorouxii*. Por el contrario, la cepa CECT 10425, puede ser una especie híbrida entre *Z. pseudorouxii* y *Z. sapae.*

Con respecto a la especie *D. hansenii* hemos diseñado unos cebadores específicos (DhPADF/ DhPADR) y un protocolo de PCR específico, rápido, fiable y económico, utilizando como diana una región (729 pb) que presenta un 69% de identidad con el gen *PAD1* de *Saccharomyces cerevisiae.* Éstos sólo dan lugar a productos de amplificación en las cepas de *D. hansenii* pero no en *D. fabryi* ni en *D. subglobosus*. Es más, dos cepas de *D. hansenii*, identificadas mediante RFLP 5,8-S ITS rDNA, que dieron resultado negativo fueron confirmadas posteriormente como cepas *de D. fabryi*. En este trabajo demostramos que la técnica RFLP 5,8-S ITS rDNA da lugar a los mismos perfiles de restricción para las especies *D. fabryi*, *D. hansenii* y *D. subglobosus*.

Debido a su relevancia como agente deteriorante, era destacable la ausencia de un método adecuado para la tipificación de cepas de *M. guilliermondii*. En este trabajo analizamos, por un lado la combinación de las técnicas de IGS-PCR RFLP del rDNA y de RFLP del mtDNA y por otro, la detección de los microsatélites. Este último método fue muy discriminante. Existen nueve fragmentos secuenciados de *M. guilliermondii*, en los cuales hemos podido comprobar *in silico* la presencia de diecinueve microsatélites (di-, tri- , tetra) en siete de ellos para los que hemos diseñado cebadores específicos. En todas las cepas de *M. guilliermondii* se amplificaron cuatro microsatélites (sc15, sc22, sc32, sc72), un resultado que no se consigue en ninguna otra especie, incluidas las que son indistinguibles fisiológicamente (*M. caribbica* y *Candida carpophila*), ni en *D. hansenii*, otra especie también muy parecida fisiológicamente. La combinación de los cebadores (sc15F/R, sc32F/R y sc72F/R) dio lugar a un único patrón para cada una de las cepas ensayadas de Colecciones de Cultivos Tipo. Este método fue validado con cepas aisladas de diferentes alimentos y diferentes orígenes geográficos. Cuatro cepas de *M. guilliermondii* dieron resultados negativos con los cebadores específicos para esta especie (sc15F/R y sc72F/R); mediante la secuenciación de la región D1/D2 del dominio 26S del DNA ribosómico y el análisis de los perfiles de restricción (*Taq*I-5,8S ITS) pudimos comprobar posteriormente que se trataba de cepas de *M. caribbica*. Los patrones entre las cepas aisladas de diferentes nichos ecológicos no se repitieron.

Por otro lado hemos abordado la aplicación práctica de alguno de los métodos desarrollados a un caso de deterioro de yogures ecológicos industriales. Todos los yogures, enviados a lo largo de dos años por un laboratorio de control de calidad, presentaron altas cargas de

contaminación por *M. guilliermondii* y algunos de ellos estaban deteriorados (hinchados). La capacidad de *M. guilliermondii* para crecer en lactato y lactato más galactosa, fermentar glucosa y débilmente galactosa además de la relación sinérgica que establece con las bacterias lácticas (LAB) es suficiente para crecer en cualquier yogur, aunque sólo deteriora los que incluyen mermelada de fruta. Nuestro análisis demuestra que la contaminación de los yogures puede pasar desapercibida para la industria y el consumidor. Más aún, la contaminación queda más enmascarada porque la fermentación, pero no el crecimiento, se inhibe fuertemente a 8ºC.

**Conclusiones**

1. El análisis de los fragmentos de restricción de la región IGS del DNA ribosómico diseñado en esta Tesis constituye un método de tipificación de cepas discriminatorio y reproducible para las especies *Z. bailii*, *Z. mellis* y *Z. rouxii* y es fácil de implantar en la rutina de un laboratorio industrial.
2. *Z. rouxii* CECT 11923 y CECT 10425 son cepas híbridas posiblemente entre las especies *Z. rouxii* y *Z. sapae,* y *Z. pseudorouxii* y *Z.sapae* respectivamente.
3. Existen diferencias en la secuencia de la región IGS1 del DNA ribosómico entre las cepas híbridas y las que no lo son. Los cebadores específicos (HibZF/HibZR) que reconocen esta región, constituyen un método rápido y económico para la detección de cepas híbridas dentro del género *Zygosaccharomyces*.
4. Las cepas de la especie *D. hansenii* pueden ser identificadas rápida y económicamente a partir de diferentes productos alimenticios con los cebadores específicos (DhPADF/DhPADR). Éstos permiten

diferenciar esta especie de *D. fabryi* o *D. subglobosus*, que son indistinguibles fisiológicamente.

5. La detección de microsatélites constituye el mejor método de los ensayados en este trabajo y hasta este momento para la discriminación de cepas de *M. guilliermondii*. El análisis combinado de los resultados obtenidos con los tres cebadores (sc15F/R, sc32F/R y sc72F/R) reduce el tiempo y el coste. Además, permite distinguir esta especie de otras fisiológicamente idénticas como *M. caribbica o C. carpophila*.
6. *M. guilliermondii* fue la especie identificada como responsable del deterioro de los yogures ecológicos. El gas producido se debe a la fermentación del azúcar de las mermeladas añadidas. La capacidad de crecer en lactato como fuente de carbono permite que esta especie se multiplique también en los yogures naturales hasta altas concentraciones $10^6$ UFC/g sin que la industria ni el consumidor lo perciba. A 8ºC continúa el crecimiento aunque la fermentación se inhibe casi totalmente. Pensando en los consumidores inmunodeprimidos debería considerarse el control de la especie *M. guilliermondii* por razones tanto de calidad como sanitarias. La aplicación de la tipificación de cepas mediante la detección de microsatélites indica que la empresa se contaminó al menos por dos cepas, una de ellas procedente de las mermeladas de fruta.

## Summary

# "Development of molecular methods for the detection and strain typing of yeasts with industrial interest"

## Introduction and Objectives

The general objective and unifying thread of this Thesis is the development of easy, rapid and affordable molecular methods for the detection and strain typing of the spoiling or/and industrial interesting yeasts species as well to analyze their performance by applying them to a study case of organic yogurt spoilage.

The yeasts species included in this work are interesting from several points of view (food spoilage, starters, biocontrol and clinic).

In terms of spoilage ability, some of the most dangerous yeasts are found in the osmotolerant, strongly fermentative *Zygosaccharomyces* genus. This genus includes yeasts that are able to resist weak-acid preservatives such as benzoic and sorbic acids. However, together with filamentous fungi, *Z. rouxii* is involved in the early fermentation of oriental foods, contributing to their organoleptic properties. Hybrids strains have been isolated from harsh environments, as industrial miso. Artificial hybrids have even been generated. Therefore we consider that the hybrid-specific detection would be very useful. Although several typing methods, mainly for *Zygosaccharomyces rouxii*, have been developed up to this Thesis there was not one that was adequate by itself.

*Debaryomyces hansenii* is widespread in nature and has been extensively studied because of its halotolerance and potential industrial applications. It is one of the microorganisms involved in the ripening of traditional cheese and dry-cured meat products as sausages or ham. Strains that formerly were included in *D. hansenii* have been recently allocated as new

species of the genus i. e. *D. fabryi, D. subglobosus*. They show close physiological similarity to *D. hansenii,* resulting in numerous misidentifications. Identification by analysis of sequences is expensive and time consuming when it comes to large scale work. Some molecular techniques have been proposed as an alternative to traditional characterization for species but before our work was published all of them failed in affordability, reliability or accuracy.

Regarding *Meyerozyma,* it is a new yeast genus that includes the old species *Pichia caribbica* and *P. guilliermondii,* now named *Meyerozyma caribbica* and *M. guilliermondii*. *M. guilliermondii* (anamorphous *Candida guilliermondii*) is a cosmopolitan species and is commonly isolated from fruits and other food stuffs. It is also included among the 17 ascomycetous yeast species most frequently related to infections. Some strain typing methods with different approaches has been suggested, such as to relate strain differentiation with different levels of the wine spoilage 4-ethylphenol production or other that distinguish strains isolated from clinical samples from those that are not, with variable results. Some authors showed that in fact it did not discriminate between strains but indeed between the *M. guilliermondii* and *M. caribbica* species. Given its wide area of interest, and notwithstanding several previously published methods, there was before our work a lack of reliable and accurate approaches for *M. guilliermondii* genotyping.

The specific **objectives** of this Thesis were:

1. To develop a single typing method for rapid strain level discrimination for several species of the *Zygosaccharomyces* genus.
2. To clarify the hybrid nature of the *Z. rouxii* strains CECT 11923 and CECT 10425.
3. To develop a molecular method for the detection of *Zygosaccharomyces* hybrids strains.
4. To develop a fast an affordable specific identification method for the *D. hansenii* yeast species.
5. To develop a single typing method for intra-specific discrimination of *M. guilliermondii* strains.
6. To analyze the industrial performance of some of these methods by applying them to a case study of spoilage of organic yogurts due to *M. guilliermondii*.

**Results and Discussion**

Regarding the *Zygosaccharomyces* genus, we have evaluated in this work the usefulness of the IGS-PCR RFLP rDNA as a method for strains typing. The amplicon size of IGS region of the rDNA was different in seven out of nine species studied. Within the *Zygosaccharomyces* genus the IGS size range from 4200 bp (*Z. mellis* and *Z. rouxii*) up to 7000 bp in *Z. kombuchaensis.* A modification of the method previously described in our laboratory made it possible to amplify IGS regions with such high size. None of three endonucleases (*Hap*II, *Hha*I and *Mbo*I), could be used independently for strain discrimination, but considering the three endonucleases we obtained a variability of 100% for *Z. mellis* and *Z. rouxii*

strains and up to 70% for *Z. bailii*. All *Z. rouxii* strains presented a set of common bands. The analysis of the RFLP patterns enables us to detected two misidentified *Z. mellis* strains (CBS 711 and CBS 7412) which had the characteristic bands of *Z. rouxii* strains. The sequence analysis of domain D1/D2 26S rDNA and the 5.8-ITS rDNA region confirmed these strains as *Z. rouxii*. Unlike previously reported methods, that need a combination of several typing techniques, the one we propose is a single method that permit a reliably strain differentiation.

This method grouped the *Z. rouxii* strains CECT 11923 and 10425 in a separate cluster together with three certified *Zygosaccharomyces* hybrid strains (NCYC1682, NCYC3060 and NCYC1682). Therefore, we tried to highlight the nature of these strains. The intergenic spacer 1 (IGS1-rDNA) showed differences in size between the strains assayed, ranging from 1300 bp to 1600 bp and presented a high polymorphism between strains and within the different clones of each strain studied. In order to avoid misinterpretation caused by results due to one or both parental species and given the recently recognized important industrial role of hybrids, in this work we have developed a specific PCR assay and a pair of hybrid-specific primers, HibZF/HibZR. This primer pair recognizes specific sequences found in the hybrid strains IGS1-rDNA. Positive amplicons were only obtained for the *Zygosaccharomyces* sp. hybrids included in this study as well as for the CECT 11923 and CECT 10425 strains. Based on species-specific primers, the D1/D2 sequencing and the divergent copies of the 5.8S ITS sequencing results, we concluded that the strain CECT 11923 could be a hybrid species between *Z. rouxii* and *Z. pseudorouxii*, whereas, the strain CECT 10425 could be a hybrid species between *Z. pseudorouxii* and *Z. sapae*.

With respect to the *D. hasenii* species, we have developed specific primers (DhPADF/DhPADR) and a rapid and affordable PCR assay for *D. hansenii* strains using as target a putative homologue (69% identity) *PAD1* region (729 bp) present in this species. These primers are species-specific for *D. hansenii* strains and gave negative results in closely related species such as *D. fabryi* and *D. subglobosus*. This PCR method permitted us to detect two *D. fabryi* strains, identified by the analysis of 5.8S-ITS rDNA RFLPs as *D. hansenii*. In this work we confirm that the 5.8S-ITS rDNA RFLP produces the same profiles in *D. hansenii*, *D. fabryi* and *D. subglobosus* and therefore lead to misidentifications.

Is worth noting, the lack of suitable methods for *M. guilliermondii* strain typing. In this work we have analyzed the combination of the IGS–PCR RFLP and RFLP-mtDNA methods, as well as the use of microsatellites markers in this species. The best result was obtained by microsatellite markers. Seven of the nine scaffolds published presented microsatellites up to 19 (di-, tri- and tetra- repetition) and we developed primer pairs for all of them. The *M. guilliermondii* strains analyzed in this work were amplified with all four microsatellite markers assayed in this work (sc15, sc22, sc32, and sc72). All the none-*M. guilliermondii* strains gave negative results, even for the physiologically similarly species such as *D. hansenii*. The physiologically indistinguishable *M. caribbica* and *C. carpophila* failed in some of them. Different patterns were obtained for all *M. guilliermondii* Type Culture Collection strains with the combination of three microsatellite markers (sc15F/R, sc32F/R and sc72F/R). This method was validated on strains from different food stuffs and from different geographic origin. Four *M. guilliermondii* strains did not amplify with two species-specific primer pair (sc22F/R and sc72F/R). Sequence analysis of

domain D1/D2 26S rDNA and *Taq*I-5.8S ITS confirmed them as *M. caribbica* strains. These results showed that no pattern is repeated between the different environmental niches.

To analyze the performance of some of the developed methods they were applied to a case study of contaminated organic yogurts, sent to us by an independent quality control laboratory along two years. All yogurts were heavily contaminated by *M. guilliermondii* but only some of them were spoiled by gas swelling. The ability of *M. guilliermondii to* grow on lactate and lactate plus galactose, ferment glucose and sucrose and weakly galactose and the synergist relation established with lactic acid bacteria, enables it to grow in any yogurt, although only those with added jam were spoiled due to the fermentation of the sucrose. So, contamination of plain yogurt would not be detected neither by the industry nor the consumer. Moreover, contamination could be masked (no spoiling detected) because fermentation, but not growth, was strongly inhibited at 8ºC.

**Conclusions**

1. PCR-RFLP analysis of the IGS region of rDNA constitutes a reproducible typing method for the *Z. bailii*, *Z. mellis* and *Z. rouxii* species. The method is easy to implant in the routine of an industrial laboratory.
2. *Z. rouxii* CECT 11923 and CECT 10425 strains possibly are hybrids between *Z. rouxii* and *Z. sapae*, and between *Z. pseudorouxii* and *Z. sapae* respectively.
3. Hybrids and non-hybrids strains present differences on the IGS1-rDNA sequence. The hybrid-specific primers (HibZF/HibZR) that

recognize this region constitute a rapid and affordable method for the detection of *Zygosaccharomyces* hybrid species.

4. Primers developed in this work (DhPADF/DhPADR) constitute a rapid and affordable method that permits the identification of *D. hansenii* among all the strains isolated from different foodstuff. They permit the differentiation of this species from physiologically indistinguishable species such as *D. fabryi* and *D. subglobosus.*
5. The detection of microsatellites of *M. guilliermondii* yeast species selected here constitutes the best method for the discrimination at intraspecific level proven in this work and up to now. The combined fragment analysis of the three primers (sc15F/R, sc32F/R and sc72F/R) reduces time and price. This method also enable us to differentiate between physiologically identical species such as *M. guilliermondii*, *M. caribbica* and *C. carpophila*
6. *M. guilliermondii* yeast species was responsible for the organic yogurts spoilage. We have proven that the gas spoilage of the analyzed organic yogurts was due to the fermentation of the sugar present in fruit jam. The ability to growth using lactate as carbon source permits this species to reach up to $10^6$ CFU/g without being perceived neither by the industry nor the consumer. At 8ºC yeast growth continues although, in the presence of fermentable sugars, fermentation was almost completely inhibited. Considering consumers with compromised immunity, yeasts as *M. guilliermondii* should be controlled throughout the whole production process for quality and safety reasons. The implementation of microsatellites typing method suggests that the industry was contaminated at least by two strains; one of them had its origin in the fruit jam.

# Capítulo 1

**Introducción y Objetivos**

Las levaduras son microorganismos importantes para el ser humano ya que desde tiempos ancestrales se usan en la producción de alimentos tan cotidianos como el pan, la cerveza y el vino. El desarrollo de nuevas tecnologías hace que su importancia haya aumentado, especialmente dentro de la ciencia, la agricultura, la industria alimentaria y la medicina (Kurtzman *et al.*, 2011a) Fig. 1.1.

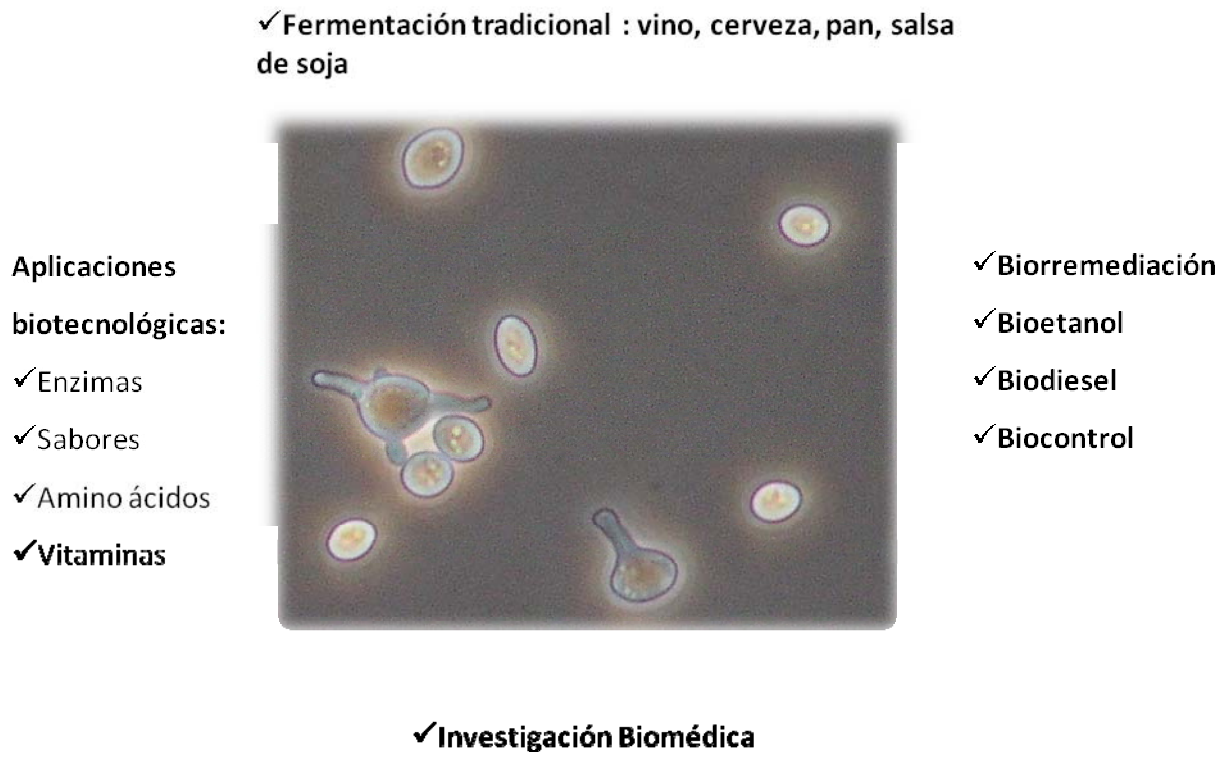


**Figura 1.1 Principales aplicaciones descritas para levaduras**.

En el mayor compendio taxonómico, "*The yeasts: a taxonomic study*" se describen 140 géneros y alrededor de 1500 especies (Kurtzman *et al.*, 2011a) que se encuentran tanto en los continentes como en ambientes marinos y presentan una amplia distribución en hábitats muy diversos. Las levaduras, frecuentemente se aíslan de sustratos naturales

como el suelo, las plantas, las frutas, los animales o los ambientes acuáticos (Boekhout y Phaff, 2003). Algunos géneros como *Candida*, *Cryptococcus, Malassezia* y *Rhodotorula* son patógenos oportunistas y recientemente se han incluido otras especies de los géneros *Trichosporum* y *Meyerozyma* (Cooper Jr, 2011) .

Dentro de la industria alimentaria, aunque una gran parte de las levaduras son consideradas beneficiosas, a lo largo de los últimos años, se ha comprobado que algunas especies son organismos alterantes que generan importantes pérdidas económicas (Deák, 2008). Varias de las alteraciones que producen en los alimentos son consecuencia de su actividad metabólica, como por ejemplo, la producción de gas o la producción de moléculas volátiles responsables de sabores y olores desagradables, mientras que otras alteraciones son debidas a la formación de biomasa en el alimento (Casas *et al.*, 2004; Chatonnet *et al.*, 1992; Pitt y Hocking, 1997).

En la actualidad la secuencia del genoma de unas 100 especies está disponible y principalmente de aquellas levaduras con un interés genético, biotecnológico o clínico (Kurtzman, 2015).

A continuación describimos brevemente la importancia de los géneros estudiados en esta tesis:

**El género *Zygosaccharomyces* Barker (1901)**

El género *Zygosaccharomyces* se clasifica dentro del grupo de levaduras ascosporógenas, Filo *Ascomycota,* Clase *Saccharomycetes*, Orden *Saccharomycetales* y Familia *Saccharomycetaceae.* Este género fue descrito por primera vez en 1901 por Barker y desde entonces ha sufrido

varias modificaciones. Actualmente, cinco de las especies antes clasificadas en este género han sido situadas en nuevos géneros o en géneros ya existentes, como por ejemplo, *Z. microellipsoides,* hoy *Torulaspora microellipsoides*. Los nuevos géneros que se han constituído son *Lachancea y Zygotorulaspora* que acogen a las especies *Lachancea cidri y L. fermentati* por un lado *y, Zygotorulaspora florentinus y Z. mrakii* por otro (James y Stratford, 2011). Por lo tanto, en la actualidad, el género *Zygosaccharomyces* ha sido reducido a 6 especies: *Z. bailii, Z. bisporus, Z. kombuchaensis, Z. lentus, Z. mellis y Z. rouxii*.

Las características del género son las siguientes:

1. Reproducción asexual por gemación multilateral
2. Células esféricas, ovoides o alargadas
3. Pseudomicelio ausente o poco diferenciado
4. Reproducción sexual por conjugación entre una célula y su yema, o entre células independientes, donde normalmente las dos células producen ascosporas. Cada asca presenta de 1 a 4 ascosporas lisas, de globosas a elipsoidales. Las ascosporas no se liberan
5. Fermentan glucosa
6. No forman película cuando crecen en medio líquido
7. La principal ubiquinona es la CoQ-6

El neotipo del género es *Z. rouxii* y se encuentra generalmente en hábitats altamente osmóticos, las cepas de esta especie se aíslan frecuentemente de mermeladas, siropes, mazapán, vino tinto, caña de azúcar, refrescos y soja (Deák, 2008; James y Stratford, 2011). Es de destacar que esta especie, pertenece al reducido grupo responsable de la mayoría de las alteraciones debidas a levaduras descritas en alimentos procesados (Pitt y Hocking, 1997; Stratford, 2006). Además, esta especie,

dentro de las levaduras es la más osmotolerante conocida, capaz de crecer con una actividad de agua ($a_w$) de 0.62 en fructosa y de 0.65 en sacarosa/glicerol. Esta característica le permite ser el agente alterante de alimentos, que en principio, no son adecuados para el desarrollo de microorganismos (Deák, 2008; James y Stratford, 2003). Además, algunas cepas son capaces de resistir elevadas concentraciones de los conservantes alimentarios denominados genéricamente como sorbatos (E200, E201, E201, E202 y E203). Algunas cepas degradan el ácido sórbico a 1.3-pentadieno, un compuesto volátil con olor a petróleo (Casas *et al.*, 2004).

A pesar de lo expuesto anteriormente, *Z. rouxii* es una levadura con importante aplicaciones biotecnológicas, en concreto en la producción de alimentos fermentados orientales (soja, miso), en los que es capaz de fermentar tanto los azucares como los aminoácidos en condiciones de alto contenido de NaCl (10-18%), dando lugar al sabor típico de los mismos (Cao *et al.*, 2010; Suezawa *et al.*, 2008; Wah *et al.*, 2013; Yuzuki *et al.*, 2015). Del mismo modo, se ha descrito que forma parte de la flora fúngica presente en el vinagre balsámico (James y Stratford, 2011; Solieri *et al.*, 2006) y se está estudiando su posible aplicación, junto con otras especie osmotolerantes, como cultivo iniciador en este producto (Solieri y Giudici, 2008), parece ser que tendría algún papel en la primera fermentación del mosto. La Tabla 1.1 refleja otras posibles aplicaciones que se están estudiando.

Por otro lado, James *et al*. (2005) describieron la existencia de híbridos naturales dentro del género *Zygosaccharomyces*. La hibridación supone un mecanismo de mejora genética y de adaptación, descrita principalmente para levaduras implicadas en procesos industriales y de importancia

clínica como algunas especies del género *Candida, Cryptococcus y Saccharomyces* (Albertin y Marullo, 2012; de Barros Lopes *et al.*, 2002; Fundyga *et al.*, 2004).

| Aplicaciones Biotecnológicas | Referencias |
|---|---|
| ✓ **1,3-propanodiol** | ✓ (Ma *et al.*, 2013) |
| ✓ **Glutaminasa** | ✓ (Iyer y Singhal, 2008; Iyer y Singhal, 2010) |
| ✓ **D-arabitol** | ✓ (Saha *et al.*, 2007) |

**Tabla 1.1 Principales posibles aplicaciones estudiadas para *Z. rouxii***

El genoma de *Z. rouxii* está secuenciado (de Montigny *et al.*, 2000) y disponible (http://www.genolevures.org/zyro.html) consta de 7 cromosomas con un tamaño de 10,4Mb.

## El género *Debaryomyces* Lodder & Kreger-van Rij (1952)

El género *Debaryomyces* pertenece, junto con el género *Meyerezyma,* al grupo de levaduras ascosporógenas, el Filo *Ascomycota,* Clase *Saccharomycetes*, Orden *Saccharomycetales* y Familia *Debaryomycetaceae.* La Familia *Debaryomycetaceae* incluye los géneros: *Debaryomyces*, *Kurtzmaniella*, *Lodderomyces*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, *Scheffersomyces*, *Schwanniomyces*, *Spathaspora*, *Wickerhamia* y *Yamadazyma* (Kurtzman, 2011b). Dentro del género hay 11 especies aceptadas (Suzuki *et al.*, 2011): *D. coudertii, D. fabryii, D. hansenii, D. maramus, D. mycophilus, D. nepalensis, D. prosopidis, D.*

*robertsiae, D. singareniensis y D. subglobosus* que presentan las siguientes características.

1. Reproducción por gemación multilateral
2. Ausencia o escaso desarrollo del pseudomicelio
3. Reproducción sexual por conjugación entre una célula y su yema, o entre células independientes. Cada asca presenta de 1 a 4 ascosporas que pueden ser globosas, ovales o lenticulares con paredes lisas o rugosas. Las ascosporas no se liberan excepto en la especie *D. udenii*
4. Fermentación de azúcares positiva, débil o ausente
5. Formación de película cuando crecen en medio líquido
6. La principal ubiquinona es la CoQ-9

*D. hansenii* es la especie tipo del género y se encuentra ampliamente distribuida en la naturaleza. Se aísla frecuentemente de suelos, aguas marinas, plantas, de diversos alimentos con un alto contenido en sal como quesos, carnes curados y salsa de soja (Büchl y Seiler, 2011; Hanya y Nakadai, 2003; Núñez *et al.*, 1996; Prista *et al.*, 2005). Esta especie no se considera patógena aunque se han descrito infecciones oportunistas debidas a la especie *Candida famata* (su forma anamorfa). Sin embargo, algunos autores consideran que puede tratarse de una confusión debida a la identificación errónea de las cepas aisladas, que en realidad corresponderían a la especie *M. guilliermondii* (Desnos-Ollivier *et al*., 2008; Kim *et al*., 2014).

El papel en la industria alimentaria es aparentemente contradictorio porque aunque *D. hansenii* se ha descrito como una especie alterante de alimentos como productos lácteos, carne procesada, alimentos fermentados y conservados con ácidos orgánicos, o alimentos con

elevado contenido en sal (Arroyo-Lopez *et al.*, 2008; Casas *et al.*, 2004; Deák, 2008; Pitt y Hocking., 1997; Sanz *et al.*, 2005; Wrent *et al.*, 2003), debido a sus capacidades proteolítica y lipolítica parece influir positivamente en las características organolépticas durante la maduración y secado de productos cárnicos (Andrade *et al.*, 2010; Cano-Garcia *et al.*, 2013; Durá *et al.*, 2004; Flores *et al.*, 2004). A su vez, está bien documentado su importante papel como parte fundamental en la flora fúngica de una gran variedad de quesos, propiciando que se proponga su utilización como cultivo iniciador en los quesos, donde contribuye a la formación de la capa superficial y a la desacidificación. Además, las actividades proteolítica y lipolítica favorecen el desarrollo del aroma del queso (Bonaiti *et al.*, 2004; Ferreira y Viljoen, 2003; Fleet, 1990; Padilla *et al.*, 2014). *D. hansenii* es una especie muy osmotolerante (4 M NaCl), esta propiedad supone varias ventajas para su aplicación en la industria biotecnológica ya que puede ser inoculada en medios de cultivo que son más resistentes a la contaminación, aumenta el rendimiento y, ambos factores contribuyen a una reducción del coste (Breuer y Harms, 2006). La Tabla 1.2 refleja las principales aplicaciones biotecnologías descritas para esta especie.

Por otro lado, el uso de cepas perteneciente a *D. hansenii* como agente de control biológico ha sido estudiada por varios autores, principalmente frente al género *Penicillium* en diferentes frutas como manzanas, limones y uvas (Hernandez-Montiel *et al.*, 2010; Stevens *et al.*, 1997; Wilson y Chalutz, 1989). Recientemente ha sido descrita su posible aplicación como antagonista frente a hongos contaminantes de productos lácticos y jamón curado (Andrade *et al.*, 2014; Liu y Tsao, 2009; Núñez *et al.*, 2015).

| Aplicaciones Biotecnológicas | Referencias |
|---|---|
| ✓ β-glucosidasa | ✓ (Jolly *et al.*, 2014; Rosi *et al.*, 1994; Strauss *et al.*, 2001; Yanai y Sato, 1999) |
| ✓ Xilitol | ✓ (Cruz *et al.*, 2000; Dominguez *et al.*, 1997; Parajó *et al.*, 1997; Pérez-Bibbins *et al.*, 2013) |
| ✓ Arabitol | ✓ (Koganti *et al.*, 2011; Koganti y Ju, 2013) |
| ✓ Riboflavina | ✓ (Burgess *et al.*, 2009; Stahmann *et al.*, 2000) |

**Tabla 1.2 Principales aplicaciones biotecnológicas descritas para la especie *D. hansenii***

Además, los estudios llevados a cabo por Gil- Serna *et al*. (2011) y Andrade *et al.* (2014) demuestran que las cepas de *D. hansenii* estudiadas no solo disminuyen el crecimiento del hongo sino también disminuyen la concentración extracelular de ocratoxina, un importante grupo de micotoxinas.

El genoma de *D. hansenii* CBS $767^T$ se ha secuenciado (Lepingle *et al.*, 2000) y está disponible (http://www.genolevures.org/deha.html). Su genoma está constituido por 7 cromosomas con un tamaño total de 12,2 Mb, excluyendo el DNA ribosómico.

## El género *Meyerozyma* Kurtzman & M. Suzuki (2010)

El género *Meyerozyma* incluye especies anteriormente clasificadas en el género *Pichia*. Pertenece al grupo de levaduras ascosporógenas del Filo *Ascomycota,* Clase *Saccharomycetes*, Orden *Saccharomycetales* y Familia *Debaryomycetaceae*. La posición taxonómica de los diferentes géneros está basada en los resultados del análisis de las secuencias de los genes LSU y SSU del rDNA y en la presencia de la ubiquinona CoQ-9 (Kurtzman y Suzuki, 2010).

Dentro del género *Meyerozyma* se reconocen hoy en día dos especies fenotípicamente idénticas, *M. guilliermondii* y *M. caribbica* que presentan las siguientes características:

1. Reproducción vegetativa por gemación multilateral
2. Producción de pseudomicelio y ausencia de hifas verdaderas
3. Reproducción sexual mediante la formación de ascosporas dentro de ascas no conjugadas. Cada asca presenta entre 1 y 4 ascosporas que pueden ser ovales o con forma de sombrero
4. Fermentación de una gran variedad de azucares

Las cepas de *M. guilliermondii* han sido aisladas de diversos sustratos naturales como las frutas, el suelo, las plantas, larvas de insectos o alimentos (Aryuman *et al.*, 2015; Corte *et al.*, 2015; Deák, 2008). Además, es considerada un patógeno oportunista en animales entre los que se incluyen los humanos (Pfaller *et al.*, 2006). Aunque *M. guilliermondii* se aísla frecuentemente de fruta deteriorada y presenta actividad fermentativa, no se ha podido demostrar que sea la responsable de su descomposición (Deák, 2008). Por otro lado, Días *et al*. (2003) incluyó a *M. guilliermondii* entre las levaduras productoras de aromas

indeseables en vinos, en particular del compuesto 4-etilfenol y sus resultados fueron respaldados por los trabajos de Martorell *et al*. (2006) y Lopes *et al*. (2009).

Desde el punto de vista biotecnológico *M. guilliermondii,* se incluye junto con *S. occidentalis*, *D. subglobosus*, *D. hansenii* y *C. albicans* en las levaduras flavogénicas que presentan interés industrial en la superproducción de riboflavina (Vitamina-$B_2$). Además esta especie ostenta otras características de interés industrial como, por ejemplo, la capacidad de convertir xilosa en xilitol, un edulcorante sustitutivo de la sacarosa (Boretsky *et al.*, 2007; Kurtzman, 2011a; Papon *et al.*, 2013). A diferencia de otras especies del grupo de "levaduras flavogénicas", *M. guilliermondii* es heterotálica y se encuentra tanto como diploide o haploide (aunque solo se han aislado formas haploides), lo que facilita el cruzamiento entre cepas y la formación de esporas (Boretsky *et al.*, 2007; Sibirny, 1996). Es de destacar que, debido a su halotolerancia y a la producción de glutaminasa extracelular (Aryuman *et al.*, 2015), recientemente, se ha propuesto a *M. guilliermondii* como cultivo iniciador en la fase temprana para la fermentación del moromi. También recientemente, Coda *et al*. (2013) han propuesto la utilización de esta especie como cultivo iniciador en la masa madre. Esto es debido a la producción de enzimas β-1,3- glucanasas que prolongan la vida media del pan.

En la Tabla 1.3 se muestran las principales aplicaciones biotecnológicas y de interés agrícola estudiadas para *M.guilliermondii*

| Aplicaciones biotecnologías | Referencias |
|---|---|
| ✓ Riboflavina | ✓ (Boretsky *et al.*, 2007; Sibirny, 1996) |
| ✓ Xilitol | ✓ (Granström *et al.*, 2001; Guo *et al.*, 2006; Rodrigues *et al.*, 2006; Zou *et al.*, 2010) |
| ✓ Enzimas industriales (Inulinasa, α-amilasa, α-ramnosidas) | ✓ (Acourene y Ammouche, 2012; Gong *et al.*, 2007; Rodriguez *et al.*, 2010; Zhang *et al.*, 2009) |

**Tabla 1.3 Principales aplicaciones biotecnológicas y de interés agrícola estudiadas en *M.guilliermondii.***

Del mismo modo, esta especie se encuentra también entre las propuestas como agentes de control biológico ya que actúa como antagonista de hongos filamentosos, sobre todo en fruta y verduras (Chanchalchaovivat *et al.*, 2007; Droby *et al.*, 1997; Lahlali *et al.*, 2011; Lima *et al.*, 2013; Zhao *et al.*, 2011). Entre los principales mecanismos que se han descrito en esta especie que pueden afectar al crecimiento fúngico se encuentra la capacidad de producir enzimas hidrolíticas, β-1,3-glucanasas, la competencia por espacio y nutrientes y, la inducción de la resistencia en la planta (Coda *et al.*, 2013; Droby *et al.*, 2009; Zhang *et al.*, 2011; Zhao *et al.*, 2008). Sin embargo, la utilización de *M. guilliermondii* (telemorfo de *C. guilliermondii*) como agente de control biológico no está libre de polémica por considerarse un patógeno oportunista, que por ejemplo, se considera como una de las especies emergentes que causan candidiasis en América Latina (Guinea *et al.*, 2014; Nucci *et al.*, 2013; Pfaller *et al.*, 2006) y, en la que se ha podido comprobar el desarrollo de

resistencia a antifúngicos, principalmente a fluconazol, durante los tratamientos (Pfaller *et al.*, 2006; Puig-Asensio *et al.*, 2014; Savini *et al.*, 2011).

El genoma de *M. guilliermondii* está secuenciado (http://www.ncbi.nlm.nih.gov/genome/?term=txid4929[orgn]) pero todavía no se encuentra ensamblado, su genoma es bastante pequeño (10,6 Mb, 8 cromosomas), pero se estima que consta de unos 5.920 genes que es un dato parecido al de otras levaduras del CTG clado (Papon *et al.*, 2013).

A continuación describimos brevemente las técnicas utilizadas frecuentemente para la identificación y tipificación de cepas.

**Técnicas de identificación**

Como se ha descrito en apartados anteriores las levaduras tienen un gran interés en diferentes ámbitos y es necesaria su correcta identificación. Tradicionalmente ésta se basaba en criterios morfológicos y fisiológicos de las cepas (Barnett *et al.*, 1990), que es un método laborioso, consume mucho tiempo y requiere personal experimentado para la interpretación de los resultados. Posteriormente con pruebas seleccionadas se diseñaron métodos miniaturizados, por ejemplo (O.B.I.S., Oxoid; API y VITEK, bioMérieux), o basados en el reconocimiento de alguna actividad enzimática (Chromogen ALbicans ®, Biomedics; CandiSelect, Biorad; Fluoroplate Candida®, Merck, DDM (Quirós *et al.*, 2005)). Actualmente este tipo de identificación se usa rutinariamente en laboratorios de control de calidad o en el campo clínico, en general se utiliza en laboratorios sin posibilidad de realizar análisis de secuencias u

otras pruebas moleculares. Sin embargo, la descripción morfofisiológica es necesaria para la descripción completa de una nueva especie (Kurtzman *et al.*, 2011b). Los avances en Biología molecular han permitido el desarrollo de varias técnicas para la identificación a nivel de especie y se consideran una buena alternativa a los métodos tradicionales, aunque no pueden utilizarse rutinariamente para la identificación de cualquier especie sin validación.

Las técnicas cuya utilización está más extendida actualmente se basan en la comparación de secuencias nucleotídicas de la región ribosómica (Fig. 1.2). El DNA ribosómico contiene copias múltiples repetidas en tándem, las zonas codificantes están altamente conservadas (18S, 5,8S, 26S y 5S). Dentro del gen 26S se localizan dos dominios variables D1 y D2, estos están flanqueados por unas secuencias conservadas. A partir de las mismas se ha desarrollado cebadores universales que permiten establecer relaciones filogenéticas entre diversos grupos de levaduras y al mismo tiempo, la identificación de especies (Kurtzman y Robnett, 1998; Kurtzman, 2015; O'Donnell, 1993). Por otro lado, entre los genes 18S y 26S existen regiones no codificantes, los espaciadores internos (ITS1 e ITS2) que junto con el gen 5,8S constituyen la región 5,8S-ITS. Los cebadores universales para esta región fueron desarrollados por White *et al*. (1990). Cabe destacar que recientemente esta región ha sido seleccionada como la diana de referencia para la identificación molecular de hongos (Schoch *et al.*, 2012).

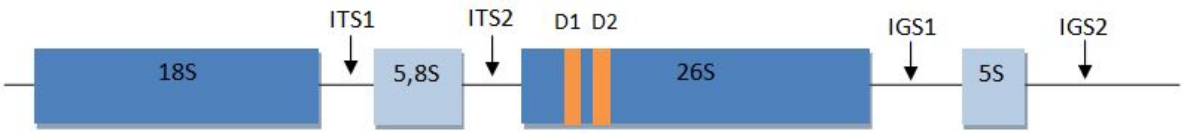


**Figura 1.2 Representación de la organización de los genes ribosómicos**

A su vez, el conocimiento de la secuencia de determinados genes permite el diseño de cebadores específicos para la detección e identificación de especies de interés. Por ejemplo, se han descrito cebadores específicos para varias especies dentro de los géneros *Brettanomyces, Saccharomyces* y *Zygosaccharomyces*, (Harrison *et al.*, 2011; Hulin *et al.*, 2014; Muir *et al.*, 2011). Gente *et al*. (2007) desarrollaron cebadores específicos para la caracterización de levaduras presentes en quesos de pasta blanda y de corteza enmohecida como *Clavispora lusitanea, Geothricum candidum, Kluyveromyces marxianus y Yarrowia lipolytica.* Hasta la realización de esta tesis no había ningún cebador específico capaz de detectar específicamente *D. hansenii*.

Otras tecnologías descritas para la identificación son:

1. **PCR-RFLPs** (*Restriction Fragment Length Polymorphism Analysis of PCR - Amplified Fragments*, polimorfismo en la longitud de los fragmentos de restricción obtenidos por PCR): consiste en digerir con endonucleasas de restricción fragmentos amplificados por PCR, utilizando principalmente la región 5,8S-ITS del rDNA. Otra región empleada con éxito ha sido la región IGS, permite diferenciar por ejemplo entre especies del género *Debaryomyces* (Cornet *et al.*, 2011; Quirós *et al.*, 2006; Romero *et al.*, 2005). No obstante, la

necesidad de una base de datos para la comparación de fragmentes hace que no sea tan útil para la identificación (Fernández-Espinar *et al.*, 2006).

2. **RAPD** (*Random Amplified Polymorphic DNA,* amplificación aleatoria de DNA polimórfico)**:** se realiza con un único cebador corto (8-10 nucleótidos) que se une al azar a sitios inespecíficos distribuidos en regiones del genoma, generando un patrón de bandas característico (Williams *et al.*, 1990). Es una técnica de fácil manejo, aplicable como método de rutina y no requiere información previa para el diseño de cebadores. Sin embargo, debido a su baja temperatura de hibridación lo convierte en una técnica poco reproducible y de difícil estandarización.

3. **AFLP** (*Amplified Fragment Length Polymorphism*, polimorfismo en la longitud de los fragmentos amplificados)**:** se basa en el uso de enzimas de restricción para digerir el DNA, seguida de la amplificación selectiva de los fragmentos generados. Se caracteriza por ser muy reproducible, fiable y no requiere un conocimiento previo del genoma (de Barros Lopes *et al.*, 1999). Sin embargo es una técnica muy laboriosa y cara, requiere secuenciadores automáticos y personal especializado. Esteve-Zarzoso *et al*. (2010) al modificar parcialmente la técnica, cambiando los cebadores y las condiciones de amplificación consiguieron reducir los fragmentos amplificados. Además, estos fragmentos se podían visualizar en geles de agarosa en lugar de los geles de poliacrilamida que se necesitaban previamente.

4. **PCR a tiempo real:** Se basa en la detección y cuantificación de un donador fluorescente. La señal del donador aumenta proporcionalmente a la cantidad de los productos amplificados, la señal es captada por un sistema de detección acoplado al termociclador. Cada vez se aplica más en la detección de levaduras alterantes de alimentos y en clínica. Además de proporcionar información también cuantitativa cabe destacar la alta sensibilidad y especificidad de esta técnica (Makino *et al.*, 2010; Tofalo *et al.*, 2012).

5. **PCR-DGGE** (*Denaturing Gradient Gel Electrophoresis,* electroforesis en gel en condiciones desnaturalizantes): mediante PCR-DGGE fragmentos de DNA del mismo tamaño pero con diferente secuencia de nucleótidos pueden ser separados en geles de poliacrilamida. Es una técnica reciente e independiente del cultivo y se aplica fundamentalmente para estudios de diversidad (Cocolin *et al.*, 2002; Muyzer *et al.*, 1993; Zhang *et al.*, 2014). Otra técnica relacionada es la de PCR-TGGE, en vez de utilizar el gradiente de desnaturalización, los fragmentos se separan por gradiente de temperatura.

6. **MALDI-TOF MS** (*Matrix - Assisted Laser Desorption /Ionization - Time Of Flight Mass Spectrometry*, Espectrometría de masas de ionización mediante laser asistida por una matriz): permite la identificación mediante la creación de un espectro, basado en el perfil de proteínas, que se compara con los espectros de referencia

en una base de datos. La técnica todavía tiene ciertas limitaciones. Una de ellas es que la base de datos de espectros no es pública y no está bien validada para levaduras, por lo que solo identifica un número relativamente pequeño de especies (Becker *et al.*, 2015; Pavlovic *et al.*, 2014).

7. **FTIR** (*Fourier Transform Infrared Spectroscopy*, espectroscopia de infrarrojos por transformada de Fourier): Esta técnica se basa en la identificación de los enlaces químicos en una molécula mediante la producción de un espectro de absorción de infrarrojos. Cada molécula orgánica tiene frecuencias características de absorción en la región del infrarrojo que dependen de la presencia de diferentes grupos funcionales en la molécula. La suma de los diferentes espectros de absorción da lugar a una huella digital, específica para cada compuesto o como en este caso para cada especie. La técnica requiere la presencia de una base de datos, es muy sensible a los cambios de temperatura, pH, composición de medios (Kumar *et al.*, 2009; Taha *et al.*, 2013).

8. **Citometría de flujo:** Las células, marcadas con fluorocromo, suspendidas en un medio pasan por delante de un laser. Los fluorocromos son excitados por el laser, emitiendo luz en diferentes longitudes de onda que permite la caracterización física y química. La ventaja de este método es que se pueden identificar rápidamente múltiples especies a partir de múltiples muestras, algo de gran interés para los laboratorios clínicos (Diaz y Fell, 2004; Page

y Kurtzman, 2005). La desventaja es el elevado coste, debido al uso de un conjunto de combinaciones de marcadores fluorescentes.

**Técnicas de tipificación de cepas**

Muchas de las propiedades que determinan si una levadura altera un alimento o si es apta para aplicaciones biotecnologías depende de la cepa y no es una característica de la especie (Albertin *et al.*, 2014; Esteve-Zarzoso *et al.*, 2010). Por ello, es muy importante contar con técnicas que permitan el reconocimiento de las cepas. Por ejemplo, para llevar a cabo un correcto seguimiento de las cepas patentadas que se inoculan en los diferentes procesos industriales. Del mismo modo, es muy importante poder demostrar el origen de una cepa que produce una alteración de un determinado alimento (*trazabilidad*). De ahí surge la necesidad de poder caracterizar a nivel de cepas. Hay varias técnicas que tratan de solucionar este problema, algunas de las que hemos mencionado como técnicas de identificación también se emplean para la tipificación con mayor o menor éxito, como RAPD, AFLP y IGS-PCR RFLP y FTIR (Appel y Gordon, 1995; Bhardwaj *et al.*, 2007; Cano-Garcia *et al.*, 2013; Corte *et al.*, 2015; Del Bove *et al.*, 2009; Esteve-Zarzoso *et al.*, 2010; Pelliccia *et al.*, 2011; Wrent *et al.*, 2010). No obstante cualquier técnica de tipificación requiere una correcta identificación previa y una validación. A continuación describimos algunas de ellas:

1. **RFLP-mtDNA** (*Mitochondrial DNA Restriction Fragment Length Polymorphism,* Polimorfismo de la longitud de los fragmentos de restricción del DNA mitocondrial): es una técnica basada en la digestión del DNA total con endonucleasas como *Hinf*I, *Hae*III y

*Rsa*I, estas enzimas reconocen gran número de dianas en el DNA genómico pero pocas en el DNA mitocondrial (Guillamón *et al.*, 1997; Querol *et al.*, 1992). Su realización es sencilla, rápida y económica además no requiere de materiales sofisticados ni personal especializado. Este método ha sido utilizado para la tipificación de varias cepas de diferentes especies, principalmente de levaduras asociadas a alimentos (Andrade *et al.*, 2006; Rodríguez *et al.*, 2011; Romano *et al.*, 1996).

2. **Amplificación de secuencias δ:** los elementos delta son secuencias de unos 300pb que flanquean los retrotransposones Ty1. En el genoma de algunas levaduras, se encuentran de forma aislada o formando parte del retrotransposon Ty1, el número y la localización de estos elementos poseen cierta variabilidad intraespecífica. Ness *et al*. (1993) desarrollaron cebadores específicos y lograron diferenciar cepas de *S. cerevisiae*. Posteriormente este método fue optimizado con nuevos cebadores que conseguían un mayor polimorfismo (Legras y Karst, 2003). La estabilidad de las secuencias δ es suficientemente alta para ser utilizada para la diferenciación de cepas de *S. cerevisiae* en la industria (Franco-Duarte *et al.*, 2011). Aunque hay descritas la presencia de retrotransposones en varias especies de levaduras esta técnica se emplea principalmente para *S. cerevisiae*. Aun así, presenta desventajas: ya que la baja temperatura de hibridación hace que aparezcan bandas inespecíficas y la concentración del DNA es de gran influencia en el resultado obtenido y, por tanto, la comparación

entre laboratorios es complicada (Fernández-Espinar *et al.*, 2006).

3. **Minisatélites y Microsatélites**: son secuencias repetidas en tándem (Fig. 1.3). Los minisatélites presentan entre 7-100 nucleótidos, que pueden representar una longitud total de 500 a 30.000 nucleótidos. Los microsatélites son secuencias cortas de uno a seis nucleótidos y pueden suponer entre 100 y 500 nucleótidos.

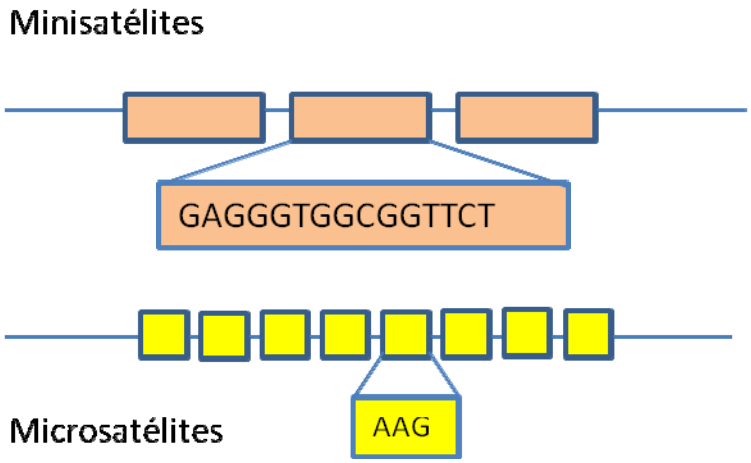


**Figura 1.3 Representación de la organización de minisatélites (repetido 3 veces) y microsatélites (repetido 8 veces).**

Los minisatélites se sitúan principalmente en los telómeros o en sus proximidades mientras que los microsatélites se sitúan por todo el genoma. Los últimos habitualmente se encuentran en zonas no codificantes del DNA, aunque esta descrito su presencia en regiones codificantes (Li *et al.*, 2004), son codominantes (permiten diferenciar entre haploide, diploide etc..) y altamente polimórficos por su alta tasa de mutación (Litt

y Luty, 1989; Miah *et al.*, 2013; Prigione *et al.*, 2004). Se ha descrito la presencia tanto de minisatélites como de microsatélites en los genomas de vertebrados, insectos, plantas y hongos, aunque el tipo de repeticiones y su frecuencia varía según las especies (Li *et al.*, 2004; Lim *et al.*, 2004). Ambos métodos pueden utilizarse con dos abordajes diferentes por un lado, su utilización como cebadores específicos ($(GACA)_4$, M13) y por otro, la amplificación especifica de los mismos. En levaduras el abordaje más utilizados es el primero (Andrade *et al.*, 2006; Canonico *et al.*, 2015; Corte *et al.*, 2015; Vigentini *et al.*, 2014; Walczak *et al.*, 2007). Mientras el segundo abordaje, hasta el momento, sólo se ha utilizado en unas pocas especies de levaduras como *B. bruxellensis, C. albicans, S. cerevisiae y S. uvarum* (Albertin *et al.*, 2014; Antonangelo *et al.*, 2013; Bonfim-Mendonca Pde *et al.*, 2013; Zhang *et al.*, 2015).

4. **MLST** (*MultiLocus Sequence Typing*, tipificación de secuencias multilocus): Este método permite diferenciar cepas de una misma especie, mediante la secuenciación de DNA de fragmentos interno de 6 a 8 genes de mantenimiento. Así cada muestra se caracteriza por las secuencias únicas de alelos en cada uno de los genes utilizados, lo que constituye su perfil alélico o secuencia tipo (ST); a cada secuencia única del gen se le asigna un número particular con el que será comparado cualquier nuevo aislamiento (Fig. 1.4). Esta técnica se utiliza principalmente para la tipificación de especies bacterianas, pero se ha aplicado también en levaduras como *C. albicans, C.*

*neoformans y S. cerevisiae* (Da Matta *et al.*, 2010; Danesi *et al.*, 2014; Wu *et al.*, 2015). Actualmente existen bases de datos para *C. albicans y C. neoformans*.

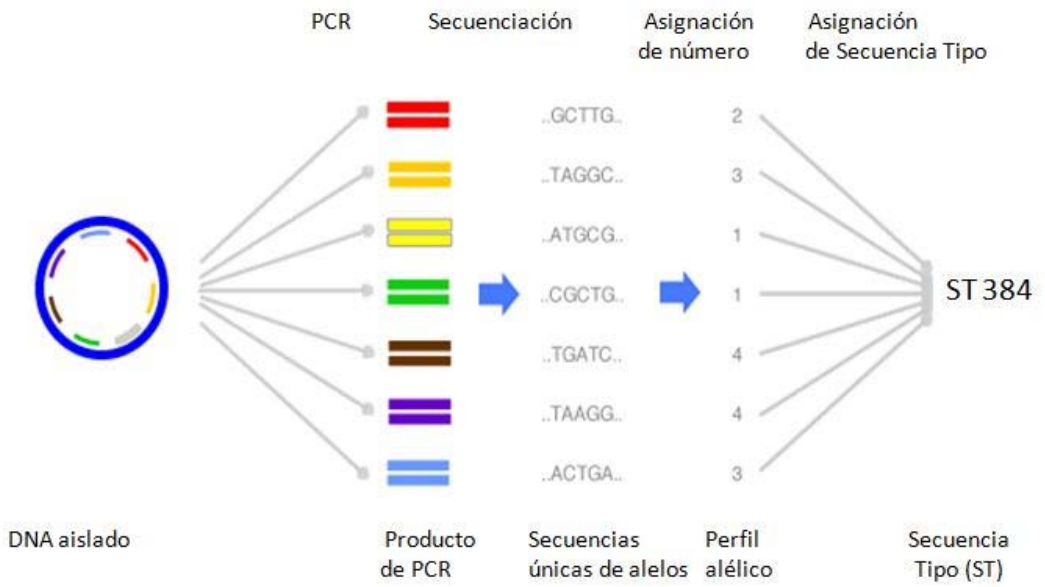


**Figura. 1.4 Representación de la técnica MLST**

## Justificación del trabajo y objetivos

En los últimos años la investigación en nuestro laboratorio se ha centrado en diversos aspectos relacionados con el crecimiento y supervivencia de las levaduras en ambientes extremos (de Silóniz *et al.*, 2002), el estudio de levaduras deteriorantes de alimentos y bebidas (Casas, 1999), en el diseño de métodos de detección (Quirós *et al.*, 2005; Quirós *et al.*, 2006; Quirós *et al.*, 2008; Romero *et al.*, 2005), en la descripción matemática de su comportamiento (Gil de Prado *et al.*, 2014) y en la elaboración de modelos predictivos (Rivas *et al.*, 2014). Dentro de los géneros que han centrado nuestra investigación, el género

*Zygosaccharomyces*, que como ya hemos comentado, es uno de los más peligrosos desde el punto de vista del deterioro, ha sido objeto de estudio en nuestro grupo sobre todo desde la perspectiva de la resistencia a conservantes (Casas *et al.*, 2004; Quintas *et al.*, 2005) y del deterioro de alimentos con $a_w$ intermedia (IMFs) (Wrent *et al.*, 2003). La prevalencia de la especie *Z. rouxii* en las industrias con las que hemos colaborado y la ausencia de un método adecuado de tipificación de cepas nos impulsó a tratar de desarrollar un método para ello. Además, en función de algunos resultados obtenidos previamente en nuestro laboratorio (Quirós *et al.*, 2006; Romero *et al.*, 2005) nos planteamos la hipótesis de que el análisis del polimorfismo de los fragmentos de restricción de la región IGS podría ser un método útil.

Por otro lado, *D. hansenii* es una especie que también ha sido objeto de nuestro estudio en el pasado (Quirós, 2005). Con el diseño de un método molecular de diferenciación de especies del género *Debaryomyces* (Quirós *et al.*, 2006) pudimos comprobar que las cepas de *D. hansenii* var. *hansenii* presentaban patrones claramente diferentes de los de *D. hansenii* var. *fabryi* y de otras cepas de *D. hansenii*. Con la reestructuración del género, llevada a cabo recientemente (Suzuki *et al.*, 2011), basada en diversos estudios genéticos (Kurtzman y Suzuki, 2010; Quirós *et al.*, 2006), las dos variedades de *D. hansenii* pasan a ser dos especies diferentes: *D. hansenii* y *D. fabryi* y las cepas de *D. hansenii*, que presentaban un patrón diferente con nuestro método, a la especie *D. subglobosus.* Las tres especies son muy difícilmente distinguibles fisiológicamente e imposible, como hemos podido demostrar en esta Tesis, con algunas de las técnicas moleculares que se utilizan rutinariamente en los laboratorios (RFLP 5,8S-ITS rDNA). La identificación

exacta es muy importante, entre otros motivos, porque uno de los principales factores que determina la validez de un estudio es la correcta identificación, ha sido, por tanto, nuestro objetivo el proporcionar una herramienta para la detección de *D. hansenii*, que es una especie de gran interés industrial como se expuso previamente, y de fácil ejecución en la industria. Los resultados obtenidos en nuestro laboratorio, en un contexto diferente al de esta Tesis, sobre la secuencia de genes que codifican para proteínas implicadas en la descarboxilación de sorbatos, *serendipicamente* nos permitieron plantear la hipótesis de que uno de los genes estudiados podría ser una buena diana.

Finalmente, a petición de un laboratorio de control de calidad, otro de nuestros objetivos ha sido abordar un problema de deterioro que resultó ser causado por *M. guilliermondii,* para la que realmente no existía hasta la realización de esta Tesis un buen método de tipificación de cepas. La hipótesis fue que podría ser adecuado algunos de los siguientes métodos: polimorfismo de los fragmentos de restricción de la región IGS, polimorfismo de los fragmentos de restricción del DNA mitocondrial y análisis de microsatélites.

Por lo tanto, el **objetivo general** de esta Tesis es, por un lado, el desarrollo de métodos que faciliten la detección de especies y que sean relativamente sencillos para su utilización en los laboratorios de las industrias y por otro, el desarrollo de métodos adecuados para la tipificación de cepas. Este abordaje, se ha llevado a cabo en las tres especies mencionadas anteriormente (*Z. rouxii*, *D. hansenii* y *M. guilliermondii*) cuyo interés en la industria se ha explicado en este capítulo de Introducción.

Atendiendo a estos antecedentes y al objetivo general expuesto anteriormente, en esta Tesis nos propusimos los siguientes **objetivos específicos**:

1. Desarrollar un método de tipificación que por sí sólo permita la diferenciación de cepas del género *Zygosaccharomyces*, con especial énfasis para la especie *Z. rouxii*.
2. Dilucidar él posible origen híbrido de las cepas de *Z. rouxii* (CECT 11923 and CECT 10425) ya que los resultados obtenidos en el objetivo 1 nos animaron a plantear esta hipótesis.
3. Desarrollar un método molecular para la detección de cepas híbridas de *Zygosaccharomyces.*
4. Desarrollar un método rápido y económico para la detección específica de *D. hansenii*.
5. Desarrollar un método para la tipificación intraespecífica de la especie *M. guilliermondii*.
6. Estudiar y analizar la aplicación de algunos de estos métodos en un caso de deterioro de yogures ecológicos causado por *M. guilliermondii*.

# Capítulo 2

**Strain typing of *Zygosaccharomyces* yeast species using a single molecular method based on polymorphism of the intergenic spacer region (IGS)**. Wrent *et al.* 2010. International Journal of Food Microbiology 142, 89-96. doi:10.1016/j.ijfoodmicro.2010.06.007

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# Strain typing of *Zygosaccharomyces* yeast species using a single molecular method based on polymorphism of the intergenic spacer region (IGS)

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ABSTRACT

Unlike previously reported methods that need a combination of several typing techniques, we have developed a single method for strain typing of the *Zygosaccharomyces bailii*, *Z. mellis* and *Z. rouxii* spoilage species. Strains belonging to other species have also been included for comparison. We have demonstrated that the IGS-PCR RFLP method has a high discriminative power. Considering the three endonucleases used in this work, we have obtained a variability of 100% for *Z. mellis* and *Z. rouxii* strains and up to 70% for *Z.bailii*. We have also detected two misidentified *Z. mellis* strains (CBS 711 and CBS 7412) which have RFLP patterns with a set of bands characteristic of *Z. rouxii* strains. Sequencing of 26S rDNA D1/D2 domains and the 5.8-ITS rDNA region confirmed these strains as *Z. rouxii*. The method also groups three certified hybrid strains of *Zygosaccharomyces* in a separate cluster.



## 1. Introduction

It has been shown that yeasts are involved in the spoilage of an extensive range of foods according to their metabolic and physiological capabilities (Stratford, 2006). However, in order to establish contamination sources during food processing and thus avoid economic losses, it is essential not only to identify which species are present but also to discriminate them at strain level.

In terms of spoilage ability some of the most dangerous yeasts are found in the *Zygosaccharomyces* genus. This genus includes osmotolerant, strongly fermentative yeasts that are able to resist weak-acid preservatives such as benzoic and sorbic acids (Casas et al., 2004). These physiological characteristics are responsible for their well-known ability to cause spoilage (Stratford, 2006). Among these yeasts are *Z. mellis*, isolated from honey, syrups and from low $a_w$ products in general (Stratford, 2006), and the *Z. rouxii* and *Z. bailii* species, commonly found in the food and drinks industries. Although the *Z. rouxii* species is involved in production of food such as miso and traditional balsamic vinegar (Solieri and Giudici, 2008), it is also frequently isolated from low $a_w$ spoiled foods like marzipan or nougat (Casas et al., 2004; Martorell et al., 2005). According to the zymological indicators defined by Sancho et al. (2000), they are considered to be some of the most dangerous yeasts for product stability in fruit pulps and concentrates.

In order for some of the *Zygosaccharomyces* species to be strain-typed, several molecular techniques have been studied. Török et al. (1993) proposed an electrophoretic karyotyping, and Esteve-Zarzoso et al. (2003) the RFLP of mtDNA. Martorell et al. (2005) demonstrated that if the objective is to differentiate species belonging to the same genus, the best result is obtained by electrophoretic analysis. If, on the other hand, it is to characterize *Z. bailli* and *Z. rouxii* at strain level, they suggested the combination of RFLP and RAPD analysis. A combination of several typing techniques was therefore required (Maqueda et al., 2010).

The intergenic region (IGS) of rDNA has the advantage that its locus is more variable than other existing loci investigated so far (Sugita et al., 2001). Several studies have exploited this region. Sequence analysis of the IGS region permitted the separation of clinical isolates of *Cryptococcus neoformans* into two varieties (Diaz et al., 2005; Diaz and Fell, 2000; Fan et al., 1995). Strain typing of *Pichia anomala* has also been achieved by analysing the sequence of the intergenic region 1(IGS1) (Bhardway et al., 2007). Other studies have focused on discriminating strains belonging to different yeast species, such as *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* (Fell and Blatt, 1999). The Restriction Fragment Length Polymorphism (RFLP) of the IGS2 region of rDNA made it possible to differentiate between the physiologically similar dairy yeast species *Kluyveromyces marxianus* and *K. lactis* (Naumova et al., 2005). We were previously able to differentiate the *Debaryomyces hansenii* yeast species in foods through PCR-RFLP of the IGS region (rDNA) (Romero et al., 2005). This also allowed us to separate the *Debaryomyces* genus into species and varieties (Quirós et al., 2006).

The aim of this investigation is to evaluate the usefulness of PCR-RFLP analysis of the IGS region of rDNA as a single typing method for

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**Table 1**
Strains studied, their origin, source of isolation, size of the amplified IGS region and code of the RFLP pattern obtained with *Hap*II, *Hha*I and *Mbo*I endonucleases.

| Species and strains studied | Source of isolation | IGS (bp) | Patterns | | |
|---|---|---|---|---|---|
| *Zygosaccharomyces bailii* (B) | | | | | |
| CECT 1898[T] | Apple juice | 6300 | BA1 | BB1 | BC1 |
| CYC 1226 | Culture contaminant | 6300 | BA1 | BB2 | BC2 |
| CECT 1924 | Unknown | 6300 | BA1 | BB1 | BC1 |
| CECT 11042 | Grape | 6300 | BA2 | BB3 | BC5 |
| CECT 11043 | Turbid wine | 6300 | BA3 | BB4 | BC3 |
| MAY(12) | Mayonnaise | 6300 | BA4 | BB5 | BC4 |
| MAY(13) | Mayonnaise | 6300 | BA4 | BB5 | BC4 |
| *Zygosaccharomyces bisporus* (Bi) | | | | | |
| CECT 11055[T] | Beer | 5500 | BiA1 | BiB1 | BiC1 |
| CECT 11348 | Beer | 5500 | BiA2 | BiB2 | BiC2 |
| *Zygosaccharomyces cidrii* (C)[a] | | | | | |
| CECT 10657[T] | Cider | 2900 | CA1 | CB1 | CC1 |
| CECT 11349 | Cider | 2900 | CA1 | CB1 | CC1 |
| *Zygosaccharomyces fermentati* (F)[a] | | | | | |
| CECT 11056[T] | Sediment of pepermint | 2900 | FA1 | FB1 | FC1 |
| CECT 10382 | Alpechin | 2900 | FA3 | FB2 | FC2 |
| CECT 10678 | *Drosophila* sp. | 2900 | FA2 | FB2 | FC2 |
| *Zygosaccharomyces kombuchaensis* (K) | | | | | |
| CBS 8849[T] | Kombucha tea | 7000 | KA1 | KB1 | KC1 |
| *Zygosaccharomyces lentus* (L) | | | | | |
| CECT 11040 | Swiss wine yeast | 6700 | LA1 | LB1 | LC1 |
| CECT 11041 | Wine | 6700 | LA2 | LB1 | LC1 |
| *Zygosaccharomyces mellis* (M) | | | | | |
| CECT 11057 [T] | Honey | 4200 | MA4 | MB3 | MC3 |
| CBS 684 | Honey | 4200 | MA1 | MB1 | MC1 |
| CBS 711[b] | Strawberry juice | 4200 | RA19[a] | RB12 | RC24 |
| CBS 735 | Fermenting honey | 4200 | MA5 | MB5 | MC1 |
| CBS 738 | Fermenting honey | 4200 | MA1 | MB4 | MC1 |
| CBS 1091 | Honey | 4200 | MA2 | MB2 | MC2 |
| CBS7277 | Alpechin | 4200 | MA3 | MB3 | MC3 |
| CBS 7412[b] | Honey | 4200 | RA20[a] | RB13 | RC25 |
| *Zygosaccharomyces microellipsiodes* (Mi)[a] | | | | | |
| CBS 427 [T] | Apple juice | 3000 | MiA1 | MiB1 | MiC1 |
| *Zygosaccharomyces rouxii* (R) | | | | | |
| CECT 1232 [T] | Grape juice | 4200 | RA1 | RB1 | RC1 |
| CECT 1231 | Bombon | 4200 | RA9 | RB1 | RC8 |
| CECT 10132 | Unknown | 4200 | RA4 | RB7 | RC16 |
| CECT 10137 | Raisin | 4200 | RA2 | RB6 | RC14 |
| CECT 10312 | Fig cake | 4200 | RA1 | RB7 | RC5 |
| CECT 10313 | Fig cake | 4200 | RA1 | RB1 | RC5 |
| CECT 10350 | Dried fig | 4200 | RA3 | RB4 | RC7 |
| CECT 10377 | Phoenix dactilifera | 4200 | RA8 | RB2 | RC3 |
| CECT 10381 | Molasses | 4200 | RA4 | RB7 | RC15 |
| CECT 10425 | Honey | 4200 | RA7 | RB10 | RC22 |
| CECT 10427 | Honey | 4200 | RA3 | RB9 | RC9 |
| CECT 10445 | Plum jam | 4200 | RA10 | RB8 | RC6 |
| CECT 10633 | Honey | 4200 | RA17 | RB7 | RC20 |
| CECT 11121 | Grape juice | 4200 | RA2 | RB7 | RC14 |
| CECT 11136 | Grapes | 4200 | RA6 | RB3 | RC10 |
| CECT 11189 | White wine | 4200 | RA11 | RB7 | RC2 |
| CECT 11923 | Soy sauce | 4200 | RA18 | RB11 | RC23 |
| CECT 11929 | Orange and lemon juice | 4200 | RA14 | RB8 | RC17 |
| CECT 12003 | Cherry | 4200 | RA4 | RB7 | RC21 |
| CECT 12004 | Cherry | 4200 | RA12 | RB7 | RC11 |
| CYC 1484 | Unknown | 4200 | RA3 | RB5 | RC4 |
| CYC 1486 | Honey | 4200 | RA16 | RB5 | RC10 |
| CYC 1487 | Nougat | 4200 | RA13 | RB7 | RC12 |
| CYC 1488 | Honey | 4200 | RA4 | RB7 | RC9 |
| NCYC 1522 | Salty bean | 4200 | RA5 | RB6 | RC15 |
| NCYC 1682 | Miso | 4200 | RA21 | RB12 | RC24 |
| NCYC 3060 | Soy sauce | 4200 | RA21 | RB12 | RC25 |
| NCYC 3061 | Soy sauce | 4200 | RA22 | RB12 | RC26 |
| T2R | Nougat fruit | 4200 | RA4 | RB7 | RC18 |
| Bch | Chocolate bun | 4200 | RA5 | RB6 | RC18 |

**Table 1** (*continued*)

| Species and strains studied | Source of isolation | IGS (bp) | Patterns | | |
|---|---|---|---|---|---|
| *Zygosaccharomyces rouxii* (R) | | | | | |
| MAY(1) | Liquid sugar | 4200 | RA15 | RB3 | RC13 |
| MAY(15) | Liquid sugar | 4200 | RA15 | RB5 | RC15 |
| Es 14 | Marzipan | 4200 | RA4 | RB6 | RC19 |

The first letter corresponds to the species: *Zygosaccharomyces bailii* (B), *Z. bisporus* (Bi), *Z. cidri* (C), *Z. fermentati* (F), *Z. kombuchaensis* (K), *Z. lentus* (L), *Z. mellis* (M), *Z. microellipsoides* (Mi), *Z. rouxii* (R), followed by the letter corresponding to the endonucleases: *Hap*II (A), *Hha*I (B), *Mbo*I (C) and finally a number corresponding to the pattern. CBS, Centraalbureau voor Schimmelcultures, The Netherlands; CECT, Colección Española de Cultivos Tipo, Spain. The remaining strains were isolated and identified in our laboratory.

[a] *Z. cidri* and *Z. fermentati* are proposed as *Lachancea* species and *Z.microellipsiodes* as *Torulaspora species* (Kurtzman, 2003, FEMS Yeast 24,403–417).

[b] Strains identified in this study as belonging to *Zygosaccharomyces rouxii* species.

rapid discrimination at strain level for the *Z. bailii*, *Z. mellis* and *Z. rouxii* species.

## 2. Materials and methods

### 2.1. Strain and culture conditions

Fifty-nine strains belonging to the *Zygosaccharomyces* genus and one strain of *Saccharomyces cerevisiae* were used in this research. They were obtained from different Type Culture Collections or isolated in our laboratory from contaminated or spoiled products. The sources of isolation, obtained from information provided by collections or in our laboratory, are shown in Table 1. The strains were grown in Yeast Morphology Broth at 28 °C and routinely maintained on Yeast Morphology Agar (YMA) containing 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, Mitch, USA), 0.3% (w/v) proteose-peptone No.3 (Difco), 0.3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain), and 2% (w/v) agar.

### 2.2. DNA isolation, amplification protocols and RFLP analysis

DNA was isolated following the standard protocol of Querol et al. (1992), which includes a first enzymatic step to obtain spheroplasted cells, followed by the isolation and purification of DNA. Alternatively, the DNeasy plant minikit (Qiagen, Hilden, Germany) was used after the spheroplast step. The IGS region of the rDNA was amplified by PCR in a Mastercycler gradient device (Eppendorf, Germany) using CNL12 (5′CTGAACGCCTCTAAGTCAG3′) and CNS1 (5′GAGACAAGCATATGAC-TACTG3´) forward and reverse primers respectively (Appel and Gordon, 1995). The region defined by these primers spans from base position 3046–3064 on the 26SrDNA (GenBank Accession no. AY048154) to base position 37-17 on the 18S rDNA (GenBank Accession no. J01353). A set of PCR reactions were carried out in microtubes containing a master mix with a final volume of: (i) 25 µl, containing target DNA (50 ng), 2 mM Mg $Cl_2$, 1 mM dNTPs (Ecogen, Madrid, Spain), 1 µM of each primer (Sygma–Genosys, Cambridge, UK), 1U *Taq* polymerase (Ecogen) and sterilized distilled water (MO Laboratories, Inc., USA) up to final volume. The thermal cycling parameters were as follows: an initial denaturation step at 94 °C for 85 s, followed by 35 cycles of 35 s at 95 °C (denaturation), 55 s at 58 °C (annealing) and a final extension at 72 °C for 10 min as previously developed in our laboratory (Romero et al., 2005; Quirós et al., 2006). In order to improve the results the following protocol was evaluated: (ii) 25 µl containing target DNA (100 ng), 12.5 µl 2xGC buffer I or II (TaKaRa bio IncShiga, Japan), 4 µl dNTP mixture (2.5 mM each) (TaKaRa), 1,25 µl of each primer (20 mM) (Sygma–Genosys, Cambridge, UK), 0.25 µl *La Taq*GC (TaKaRa) and sterilized distilled water (MO Laboratories, Inc) up to 25 µl. PCR conditions were an initial denaturation at 94 °C for 85 s and 35 cycles of denaturation at 95 °C

for 35 s. For annealing, a gradient of 54.9 °C to 69.5 °C for 55 s was probed, followed by an extension for 10 min at 72 °C. PCR-amplified DNA fragments were separated in 1% (w/v) agarose gels (Bio-Rad), stained with 0.05% (v/v) ethidium bromide (Bio-Rad) and visualized under UV light. The 1 kb DNA ladder (MBI Fermentas) was used as a molecular size marker.

PCR amplification products from the IGS region of DNA (20 µl) were digested without further purification using *Hap*II, *Hha*I and *Mbo*I endonucleases (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Romero et al., 2005; Quirós et al., 2006). Restriction fragments were separated on 2.5% (w/v) agarose gels, stained with 0.05% (v/v) ethidium bromide and visualized under UV light. The 100 bp DNA ladder (MBI Fermentas) was used as a molecular size marker.

### 2.3. Sequence determination

An analysis of sequences was carried out only on those strains which had a doubtful species classification. The 5.8-ITS rDNA region sequences were amplified by PCR using its1 (5′-TCCGTAGGTGAACCTGCGG-3′) and its 4 (5′TCCTCCGCTTATTGATATGC3′) primers (White et al., 1990). The D1/D2 domains of the 26S rDNA were amplified using NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Kurtzman and Robnett, 1998). PCR products were cleaned using the Ultraclean™ PCR clean-up Kit (MO-BIO, Larsband, USA) and 5 µM directly sequenced using the ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Foster City, USA). Each set of sequences was aligned using the MegAlign program of DNASTAR software (Lasergene, Wisconsin, USA), with the most similar sequences obtained from the GenBank Nucleotide sequence Database by BLAST. Alignments were manually revised and edited in the insertion-deletion regions for which alignment was uncertain.

### 2.4. Data analysis

Distance analysis was performed with TREECOM v1.3b software (Van de Peer and De Wachter, 1993). Distance estimations were calculated following the method described by Nei and Li (1979). The data on RFLP patterns were coded as binary tables, with 1 representing the presence of a fragment and 0 representing the absence of a fragment. The tree was inferred by the Neighbor-Joining algorithm. The robustness of the tree was estimated with bootstrap values on 1000 replicates. *S. cerevisiae* CECT 1942$^{NT}$ was the designated outgroup in the analysis.

## 3. Results

The IGS region (rDNA) with a size lower than 4000 bp was amplified using the protocol (see method 2.2. (i) in Materials and methods). Those with a size higher than 4000 bp had to be amplified with *La Taq GC* polimerase for DNA fragments rich in G + C (GC buffer I). The annealing temperature was set at 55 °C for 55 s (see method 2.2. (ii) in Materials and methods). The PCR yielded a single fragment that ranged from 2900 bp for the *Z. cidri* (now *Lachancea cidri*, Kurtzman, 2003) species to 7000 bp for the *Z. kombuchaensis* species. As can be observed in Table 1, the size of the amplification products is identical when the strains belong to the same species.

The digestion of the IGS amplification product reveals 37 different patterns for *Hap*II, 32 for *Hha*I, and 43 for *Mbo*I enzymes (Tables 1 and 2). In general, high polymorphism was obtained for each species with the three enzymes assayed (Tables 1 and 2, Figs. 1 and 2). None of the endonuclease could be used independently to discriminate strains and species. The number of patterns varies depending on the endonuclease used. For example, for *Z. bailii* we obtained five patterns with the *Hha*I endonuclease and four with the *Mbo*I and *Hap*II endonucleases (Tables 1 and 2, Fig. 2). However, in *Z. rouxii* the best discrimination between strains was obtained with the *Mbo*I endonuclease (Tables 1 and 2, Fig. 1).

The restriction patterns obtained with all three endonucleases, as illustrated in Table 1, enable us to differentiate all the *Z. mellis* and *Z. rouxii* strains examined in this study. For example, strains that show the RA1 pattern with *Hap*II (*Z.rouxii* CECT 1232$^{T}$, CECT 10312 and CECT 10313) can be differentiated if the other two enzymes are considered, namely, RA1/RB1/RC1, RA1/RB7/RC5 and RA1/RB1/RC5 respectively.

When the strain patterns belonging to different species were analysed, together with the dendrogram (Fig. 3), some conflicting results were observed. Two strains, CBS 711 and CBS 7412, identified as *Z. mellis,* appeared inside the main cluster of *Z. rouxii*. The restriction profiles for the strains included in this cluster showed common bands with whichever endonucleases were assayed. However, the *Z. rouxii* CECT 11923 and CECT 10425 strains, together with *Z. rouxii* hybrids, appeared in a second cluster because of the lack of these common bands (Table 2). To solve this conflict, sequence analyses of ITS rDNA and the D1/D2 domains of 26S rDNA were performed (Table 3). The sequences studied showed that *Z. mellis* CBS 711 and CBS 7412 have 100% similarity with strains belonging to *Z. rouxii* species (see Table 3). Two patterns were therefore added to *Z. rouxii* strains (Table 1). The CECT 11923 and CECT 10425 strains were also confirmed as *Z. rouxii*.

## 4. Discussion

In food industries, strain typing can be considered from two perspectives: one for discriminating between different biotypes with specific properties, such as the production of volatile compounds for improving taste, ripening etc. (Andrade et al., 2009), as well as for monitoring the behaviour of a strain, for example, during the fermentation process (Suezawa et al., 2008), and the other for following one strain to determine the source of contamination, for example, to solve spoilage problems along the production chain in a specific industrial process (Martorell et al., 2005). Traceability is becoming a concept for providing safer food supplies and connecting the producer with the consumer (Regattiere et al., 2007). EC regulation 178/2002 (European Parliament, 2002) defines traceability as “the ability to trace and follow a food, feed, food-producing animals or ingredients through all stages of production and distribution”. In terms of food safety, it can tell us the history of the product. In the food spoilage context a method that attains a high grade of discrimination between strains allows us to follow the “traceable spoilage strain” along the production line. This provides the industrialist with a useful tool which may be required for legal demands.

Our results show that the PCR-RFLP-IGS analysis presents a variability of 70% for *Z. bailii* with both *Hha*I and *Mbo*I endonucleases. Meanwhile, 100% variability was obtained for *Z. mellis* and *Z. rouxii* strains when the three enzymes were analysed together. This variability is higher than what has previously been reported for some of these species using other methods (Martorell et al., 2005; Suezawa et al., 2008). Moreover, if we analyse some of the strains with the same patterns some considerations should be made. For example, in *Z. bailii* the May (12) and May (13) strains were isolated in our laboratory from the same mayonnaise sample. It is therefore possible that they come from two colonies of the same strain. The CECT 1924 and CECT 1898 strains show the same RFLP pattern. Martorell et al. (2005) had previously studied them using RFLP mtDNA and RAPDs and found that they could not be distinguished by those methods. Although both come from Japan, the origin of only one of them is known (Table 1). Based on these results we propose that both isolates belong to the same strain.

Other examples of identical RFLP pattern were found in the CECT 10657$^{T}$ and CECT 11349 strains of *Z. cidri* (now *Lachancea cidri*, Kurtzman, 2003). Both were deposited by the same author in the same year (1955) and were from the same origin: cider. Once again, we propose that both isolates belong to the same strain.

**Table 2**
Restriction fragments (bp) obtained after the digestion of the IGS region of rDNA of species belonging to the *Zygosaccharomyces* genus with *Hap*II, *Hha*I and *Mbo*I endonucleases.

| Species/No. (Code of Species) | Restriction fragments[a] (bp) | | |
|---|---|---|---|
| | Patterns Hap II (A) | Patterns Hha I (B) | Patterns Mbo I (C) |
| *Zygosaccharomyces bailii*/7. (B) | | | |
| | | BB1 (1750 + 610 + 450 + 430 + 360 + 340 + 300 + 200 + 160) | BC1 (2600 + 950 + 610 + 410 + 320 + 270) |
| | BA1 (1000 + 900 + 700 + 530 + 290 + 270 + 200 + 170 + 140) | BB2 (1900 + 1700 + 610 + 450 + 430 + 360 + 300 + 200 + 160) | BC2 (2700 + 2600 + 950 + 610 + 410 + 320 + 270) |
| | BA2 (1000 + 700 + 530 + 420 + 220 + 180 + 170 + 140) | BB3 (2300 + 2000 + 610 + 450 + 430 + 350 + 330 + 300 + 200 + 160) | BC3 (3000 + 950 + 610 + 410 + 320 + 270) |
| | BA3 (1000 + 700 + 530 + 350 + 290 + 200 + 170 + 140) | BB4 (2800 + 2500 + 610 + 450 + 430 + 360 + 300 + 200 + 160) | BC4 (1300 + 1200 + 950 + 610 + 410 + 370 + 350 + 320 + 270) |
| | BA4 (900 + 620 + 530 + 480 + 430 + 390 + 350 + 220 + 180 + 170 + 140) | BB5 (2500 + 610 + 480 + 430 + 360 + 340 + 300 + 160) | BC5 (2700 + 950 + 610 + 410 + 400 + 320 + 270) |
| *Zygosaccharomyces bisporus*/2. (Bi) | | | |
| | BiA1 (1150 + 1100 + 630 + 510 + 430 + 370 + 300 + 220 + 170 + 120 + 90) | BiB1 (1150 + 870 + 580 + 390 + 360 + 290 + 240 + 200 + 190 + 140 + 100) | BiC1 (1500 + 1150 + 680 + 630 + 400 + 330 + 270 + 230 + 150) |
| | BiA2 (1500 + 1200 + 630 + 600 + 510 + 430 + 370 + 300 + 170 + 120 + 90) | BiB2 (1150 + 870 + 580 + 480 + 390 + 360 + 290 + 240 + 200 + 190 + 140 + 100) | BiC2 (1500 + 1150 + 710 + 630 + 400 + 330 + 270 + 230 + 150) |
| *Zygosaccharomyces cidrii*/2. (C) | | | |
| | CA1 (2000 + 320 + 270 + 210 + 150) | CB1 (1180 + 760 + 730 + 300) | CC1 (1100 + 460 + 330 + 320 + 250 + 210 + 170 + 110) |
| *Zygosaccharomyces fermentati*/3. (F) | | | |
| | FA1 (1150 + 800 + 390 + 170) | FB1 (1100 + 820 + 380 + 380 + 170) | FC1 (750 + 690 + 390 + 320 + 250 + 210 + 110) |
| | FA2 (1100 + 900 + 550 + 300 + 250) | FB2 (1100 + 850 + 550 + 300 + 250) | FC2 (1000 + 570 + 390 + 320 + 250 + 210 + 110) |
| | FA3 (1500 + 600 + 320 + 270 + 210 + 150) | | |
| *Zygosaccharomyces kombuchaensis*/1. (K) | | | |
| | KA1 (1600 + 1150 + 600 + 510 + 430 + 410 + 300 + 250 + 190 + 170 + 120) | KB1 (1900 + 600 + 600 + 500 + 450 + 450 + 400 + 250 + 210 + 120) | KC1 (1750 + 1250 + 1150 + 950 + 550 + 500 + 450) |
| *Zygosaccharomyces lentus*/2. (L) | LA1 (680 + 580 + 530 + 500 + 430 + 330 + 300 + 280 + 240 + 200 + 180 + 150) | LB1 (1500 + 1300 + 900 + 600 + 580 + 500 + 420 + 300 + 270 + 200 + 150 + 90) | LC1 (1100 + 850 + 610 + 590 + 550 + 450 + 320 + 310 + 270 + 250 + 150 + 110) |
| | LA2 (730 + 580 + 530 + 500 + 430 + 330 + 300 + 280 + 240 + 200 + 180 + 150) | | |
| *Zygosaccharomyces mellis*/10. (M) | MA1 (1000 + 930 + 300 + 280 + 190 + 160 + 120 + 90) | MB1 (1750 + 900 + 800 + 400 + 250) | MC1 (1300 + 900 + 720 + 430 + 350 + 270 + 150 + 120 + 100) |
| | MA2 (1000 + 930 + 480 + 300) | MB2 (1750 + 850 + 500 + 400 + 390) | MC2 (1300 + 900 + 750 + 450) |
| | MA3 (1000 + 800 + 750 + 300 + 250) | MB3 (1300 + 680 + 530 + 450 + 380 + 310 + 210) | MC3 (1250 + 1000 + 450 + 410 + 260) |
| | MA4 (1000 + 750 + 300 + 250 | MB4 (1750 + 900 + 800 + 530 + 400 + 250) | RC24 (760 + 670 + 520 + 500 + 410 + 350 + 260 + 170) |
| | MA5 (1100 + 1000 + 930 + 300 + 280 + 190 + 160 + 120 + 90) | MB5 (1750 + 1050 + 900 + 400 + 250) | RC25 (760 + 650 + 500 + 500 + 410 + 350 + 310 + 260 + 170) |
| | RA19 (970 + 700 + 500 + 400 + 380 + 300 + 290 + 160) | RB12 (960 + 700 + 630 + 450 + 420 + 210 + 190) | |
| | RA20 (970 + 700 + 480 + 400 + 310 + 300 + 290 + 160) | RB13 (960 + 700 + 680 + 630 + 450 + 420 + 210 + 190) | |
| *Zygosaccharomyces microellipsiodes*/1. (Mi) | MiA1 (1100 + 650 + 370 + 180 + 160) | MiB1 (800 + 670 + 610 + 320 + 350) | MiC1 (800 + 550 + 370 + 270) |
| *Zygosaccharomyces rouxii*/33. (R) | RA1 (970 + 700 + 500 + 400 + 300 + 290 + 160) | RB1 (960 + 700 + 630 + 450 + 420) | RC1 (760 + 670 + 520 + 500 + 410 + 350 + 260 + 200 + 170) |
| | RA2 (970 + 700 + 480 + 400 + 300 + 270 + 250 + 160) | RB2 (960 + 700 + 600 + 450 + 420) | RC2 (760 + 520 + 410 + 350 + 260 + 170) |
| | RA3 (970 + 700 + 480 + 400 + 300 + 270 + 160) | RB3 (960 + 700 + 630 + 450) | RC3 (760 + 520 + 410 + 350 + 300 + 260 + 170) |
| | RA4 (970 + 700 + 480 + 400 + 300 + 290 + 160) | RB4 (960 + 720 + 700 + 600 + 450 + 420) | RC4 (760 + 520 + 410 + 350 + 310 + 260 + 170) |
| | RA5 (970 + 700 + 480 + 400 + 300 + 290 + 270 + 160) | RB5 (960 + 720 + 700 + 630 + 450) | RC5 (760 + 520 + 500 + 410 + 350 + 260 + 170) |
| | RA6 (970 + 700 + 480 + 400 + 330 + 300 + 270 + 160) | RB6 (960 + 720 + 700 + 630 + 450 + 420 + 210) | RC6 (760 + 650 + 500 + 410 + 350 + 260 + 170) |
| | RA7 (1200 + 700 + 410 + 380 + 320 + 210 + 180 + 90) | RB7 (960 + 740 + 700 + 630 + 450 + 420 + 210) | RC7 (760 + 650 + 500 + 410 + 350 + 290 + 260 + 170) |
| | RA8 (970 + 700 + 480 + 400 + 350 + 300 + 270 + 160) | RB8 (960 + 790 + 700 + 600 + 450 + 420) | RC8 (760 + 650 + 520 + 410 + 350 + 260 + 210 + 170) |
| | RA9 (970 + 700 + 480 + 400 + 350 + 300 + 290 + 160) | RB9 (960 + 700 + 630 + 450 + 420 + 180) | RC9 (760 + 650 + 520 + 410 + 350 + 260 + 170) |
| | RA10 (970 + 700 + 480 + 420 + 400 + 300 + 270 + 160) | RB10 (1500 + 800 + 700 + 400 + 350 + 310 + 190 + 120 + 80) | RC10 (760 + 650 + 520 + 410 + 350 + 310 + 260 + 170) |

**Table 2** (*continued*)

| Species/No. (Code of Species) | Restriction fragments[a] (bp) | | |
|---|---|---|---|
| | Patterns Hap II (A) | Patterns Hha I (B) | Patterns Mbo I (C) |
| *Zygosaccharomyces rouxii*/33. (R) | RA11 (970 + 700 + 500 + 400 + 300 + 270 + 160)<br>RA12 (970 + 700 + 500 + 400 + 300 + 290 + 200 + 160)<br>RA13 (970 + 700 + 500 + 400 + 350 + 300 + 290 + 270 + 200 + 160)<br>RA14 (970 + 700 + 500 + 450 + 400 + 300 + 290 + 160)<br>RA15 (970 + 700 + 500 + 480 + 400 + 300 + 290 + 160)<br>RA16 (970 + 700 + 500 + 480 + 400 + 370 + 300 + 290 + 270 + 160)<br>RA17 (970 + 700 + 480 + 400 + 300 + 290 + 200 + 160)<br>RA18 (1300 + 800 + 700 + 410 + 380 + 320 + 210 + 180)<br>RA21 (1300 + 730 + 700 + 410 + 380 + 350 + 320 + 210 + 180 + 160 + 90)<br>RA22 (1300 + 730 + 700 + 410 + 380 + 320 + 210 + 180 + 160 + 90) | RB11 (1500 + 850 + 700 + 400 + 350 + 290 + 120 + 80)<br>RB12 (1500 + 850 + 680 + 400 + 360 + 350 + 190) | RC11 (760 + 650 + 520 + 500 + 410 + 350 + 310 + 260 + 170)<br>RC12 (760 + 670 + 520 + 500 + 410 + 350 + 310 + 260 + 170)<br>RC13 (760 + 700 + 500 + 410 + 350 + 260 + 170)<br>RC14 (760 + 700 + 520 + 410 + 350 + 290 + 260 + 170)<br>RC15 (760 + 700 + 520 + 500 + 410 + 350 + 260 + 170)<br>RC16 (760 + 700 + 520 + 500 + 410 + 350 + 300 + 260 + 170)<br>RC17 (760 + 700 + 650 + 520 + 500 + 410 + 350 + 260 + 170)<br>RC18 (760 + 700 + 670 + 520 + 500 + 410 + 350 + 310 + 260 + 170)<br>RC19 (760 + 700 + 690 + 650 + 520 + 500 + 410 + 350 + 310 + 260 + 170)<br>RC20 (760 + 650 + 520 + 500 + 410 + 350 + 260 + 170)<br>RC21 (760 + 700 + 650 + 520 + 500 + 410 + 350 + 310 + 290 + 260 + 170)<br>RC22 (1700 + 700 + 630 + 500 + 400 + 250 + 170)<br>RC23 (1700 + 800 + 700 + 500 + 400 + 250)<br>RC24 (1700 + 800 + 750 + 700 + 650 + 500 + 400 + 270 + 170 + 160 + 130)<br>RC25 (1700 + 800 + 650 + 500 + 400 + 270 + 170 + 160 + 130)<br>RC26 (1700 + 800 + 650 + 500 + 400 + 270 + 160 + 130) |

*Z.cidri* and *Z. fermentati* are proposed as *Lachancea* species and *Z.microellipsiodes* as *Torulaspora* species (Kurtzman, 2003, FEMS Yeast 24,403–417).
[a] Some fragments could be duplicated.

One interesting result, although it was not part of our objective, was that the method enabled us to detect two misidentified strains of *Z. mellis* (CBS 711 and CBS 7412). These strains presented a restriction profile of the IGS region of rDNA which included the common bands described above for the greater part of *Z. rouxii* examined (Table 2). The D1/D2 domain 26S rDNA and ITS sequence analysis showed that

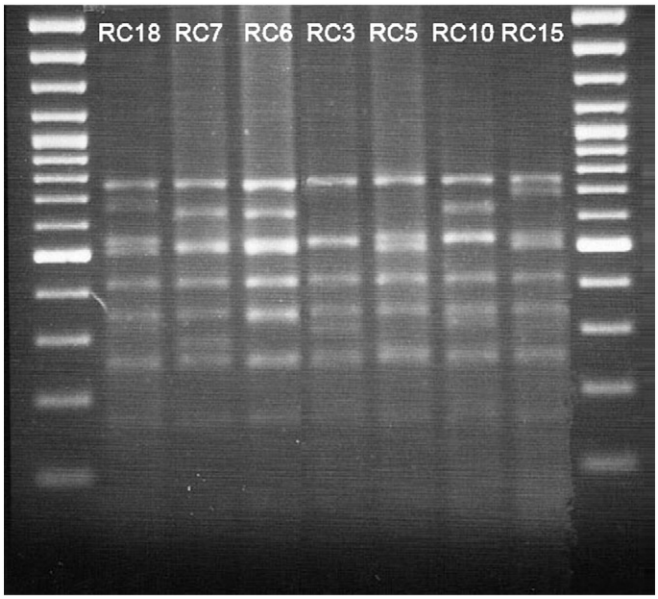


**Fig. 1.** *Zygosaccharomyces rouxii* strain typing. Some PCR-RFPLs patterns of the IGS region of rDNA with *Mbo*I endonuclease. Lanes 1 and 9 correspond to the 100 bp DNA ladder (MBI Fermentas).

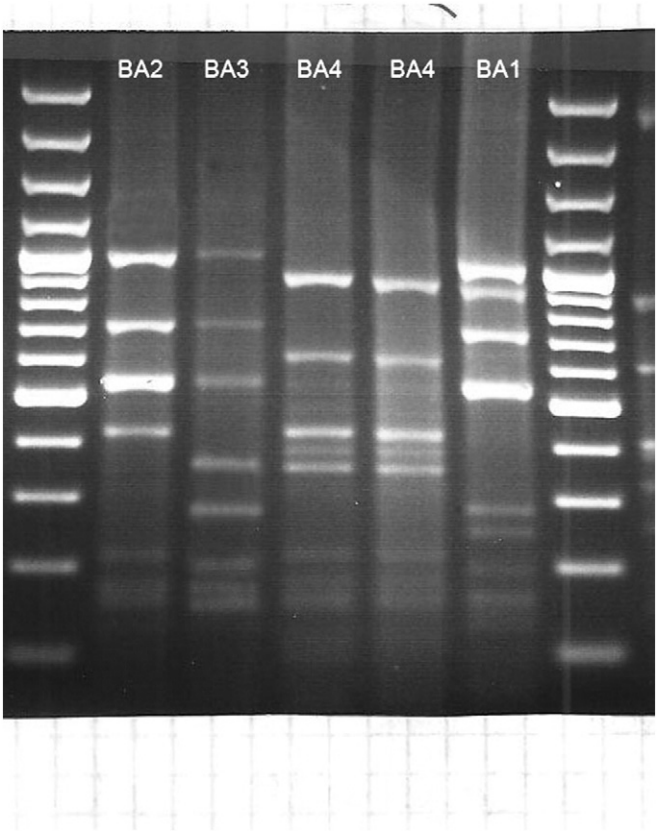


**Fig. 2.** *Zygosaccharomyces bailii* strain typing. Some PCR-RFPLs patterns of the IGS region of rDNA with *Hap*II endonuclease. Lanes 1 and 7 correspond to the 100 bp DNA ladder (MBI Fermentas).

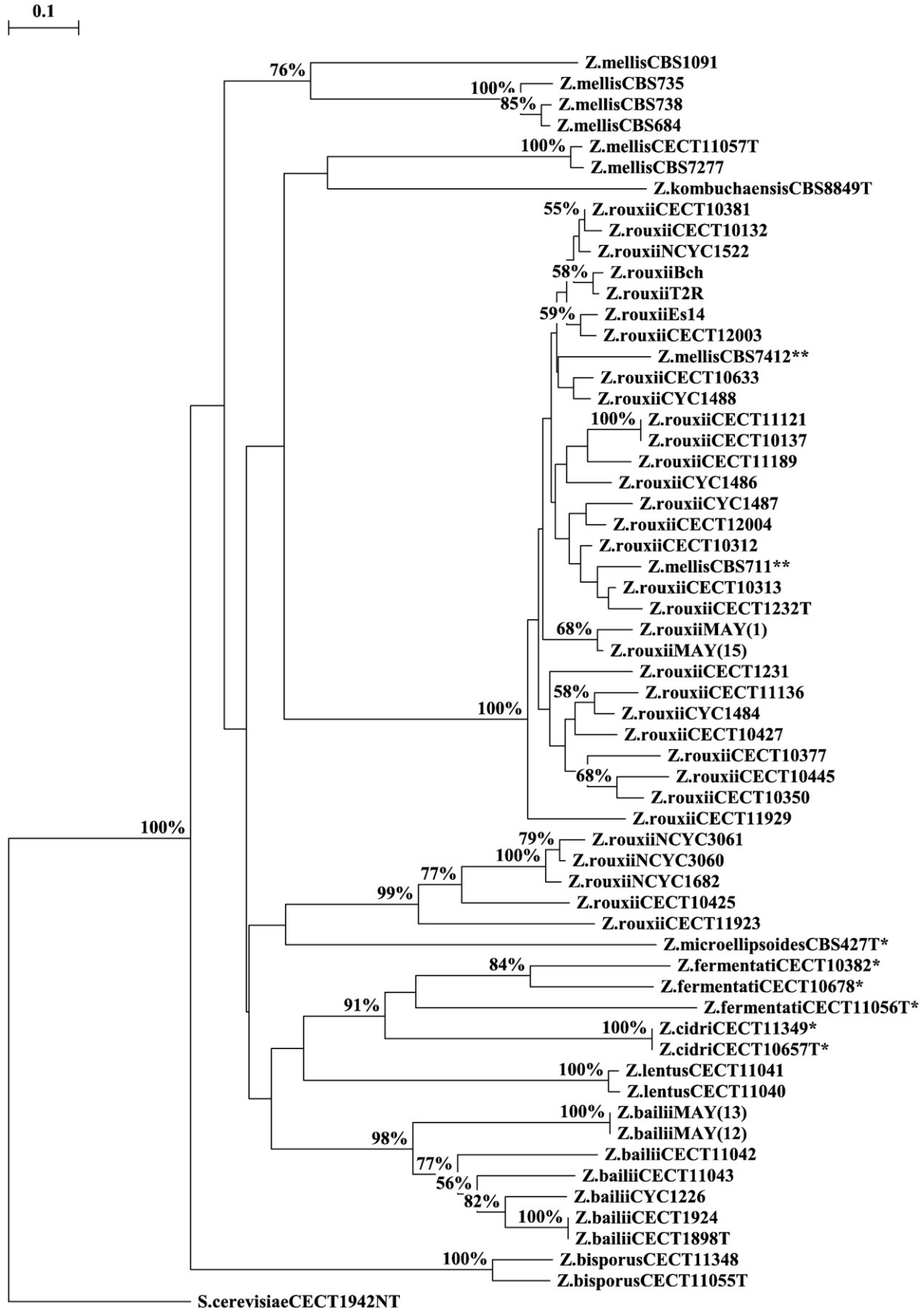


**Fig. 3.** Dendrogram based on the IGS-PCR restriction analysis of *Zygosaccharomyces* species isolated in our laboratory or from Type Culture Collections. Distance estimations were calculated following the method described by Nei and Li (1979). Robustness of the tree was estimated with bootstrap values on 1000 replicates indicated as a percentage. *Saccharomyces cerevisiae* CECT194n$^{NT}$ was used as the outgroup species. *Z. cidri* and *fermentati* are proposed as *Lachancea* species and *Z. microellipsiodes* as *Torulaspora* species (Kurtzman, 2003, FEMS yeast 24, 403–417). ** Strains identified in this study as *Z.rouxii* are shown in Table 3.

**Table 3**
GenBank accession numbers of the strains included in the study, and strains that presents a 100% similarity with them.

| Accession number | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | 5.8S-ITS (strain) | | | | | | D1/D2 26S rRNA gene (strain) | | | | | |
| *Z. rouxii* CECT 10425 | FN431888 | | | | | | FN431893 | | | | | |
| | | | | | | | AB363048.1 | (ZR510) | AB363047.1 | (ZR14) | AB363045.1 | (TSY-5) |
| | | | | | | | AB363044.1 | (TO) | AB363043.1 | (TA10101) | AB363040.1 | (No.3) |
| | | | | | | | AB363038.1 | (KS02) | AB363035.1 | (H14-8-1) | AB363033.1 | (H14-5-1) |
| | | | | | | | AB363032.1 | (H14-4-1) | AB363031.1 | (H14-1-2) | AB363030.1 | (-601) |
| | | | | | | | AB302811.1 | (IFO 1877) | AB302810.1 | (IFO 1876) | AB302807.1 | (IFO 1812) |
| | | | | | | | AB302805.1 | (IFO 0845) | AB302801.1 | (IFO 0596) | AB302800.1 | (IFO 0525) |
| | | | | | | | AB302799.1 | (IFO 0523) | AB302798.1 | (IFO 0521) | AB302794.1 | (IFO 0510) |
| | | | | | | | AB302793.1 | (IFO 0506) | AB302792.1 | (IFO 0505) | AM947682.1 | (ABT301) |
| | | | | | | | AM947680.1 | (ATCC 42981) | AM943657.1 | (ATCC 42981) | AJ966342.2 | (ABT301) |
| | | | | | | | AY524006.1 | AY524006.1 (NRRL Y-2547) | AJ555406.1 | (NCYC3042) | | |
| *Z. rouxii* CECT 11923 | FN431886 | | | | | | FN431887 | | | | | |
| | AB363062.1 | (ZR510) | AB363061.1 | (ZR14) | AB363060.1 | (ZR-1) | CU928181.1 | (CBS 732) | AB363046.1 | (ZR-1) | AB363042.1 | (SR-4) |
| | AB363057.1 | (SR-4) | AB363056.1 | (SR-2) | AB363055.1 | (KS03) | AB363041.1 | (SR-2) | AB363039.1 | (KS03) | AB363037.1 | (KS01) |
| | AB363054.1 | (KS01) | AB363053.1 | (KF-4) | AB363051.1 | (H14-7-1) | AB363036.1 | (KF-4) | AB363034.1 | (H14-7-1) | AB302813.1 | (IFO 1960) |
| | AB302834.1 | (IFO 1960) | AB302827.1 | (IFO 1130) | AB302826.1 | (IFO 0845) | AB302812.1 | (IFO 1914) | AB302809.1 | (IFO 1814) | AB302808.1 | (IFO 1813) |
| | AB302824.1 | (IFO 0596) | AB302820.1 | (IFO 0513) | AB302819.1 | (IFO 0512) | AB302806.1 | (IFO 1130) | AB302804.1 | (IFO 0740) | AB302803.1 | (IFO 0686) |
| | AB302818.1 | (IFO 0511) | AM943656.1 | (ATCC 42981) | AM943655.1 | (CBS 732) | AB302797.1 | (IFO 0513) | AB302796.1 | (IFO 0512) | AB302795.1 | (IFO 0511) |
| | AY046189.1 | (Unknown) | | | | | AB302791.1 | (IFO 0494) | AM943656.1 | (ATCC 42981) | AM943655.1 | (CBS 732) |
| | | | | | | | AM911009.1 | (YSF21) | AM911008.1 | (YSF34) | AJ783434.1 | (ESAB21) |
| | | | | | | | AJ716118.1 | (CBS 9714) | U72163.1 | (Unknown) | | |
| *Z. mellis* CBS 711 | FN431889 | | | | | | FN431892 | | | | | |
| | AB363062.1 | (ZR510) | AB363061.1 | (ZR14) | AB363060.1 | (ZR-1) | CU928181.1 | (CBS 732) | AB363046.1 | (ZR-1) | AB363042.1 | (SR-4) |
| | AB363057.1 | (SR-4) | AB363056.1 | (SR-2) | AB363055.1 | (KS03) | AB363041.1 | (SR-2) | AB303639.1 | (KSO3) | AB393037.1 | (KSO1) |
| | AB363054.1 | (KS01) | AB363053.1 | (KF-4) | AB363051.1 | (H14-7-1) | AB363036.1 | (KF-4) | AB363034.1 | (H-14-7-1) | AB302813.1 | (IFO1960) |
| | AB302834.1 | (IFO 1960) | AB302827.1 | (IFO 1130) | AB302826.1 | (IFO 0845) | AB302812.1 | (IFO1914) | AB302809.1 | (IFO1814) | AB302808.1 | (IFO1813) |
| | AB302825.1 | (IFO 0740) | AB302824.1 | (IFO 0596) | AB302820.1 | (IFO 0513) | AB302806.1 | (IFO1130) | AB302804.1 | (IFO0740) | AB302803.1 | (IFO0686) |
| | AB302819.1 | (IFO 0512) | AB302818.1 | (IFO 0511) | AM943656.1 | (ATCC 42981) | AB302797.1 | (IFO0513) | AB302796.1 | (IFO0512) | AB302795.1 | (IFO0511) |
| | AM943655.1 | (CBS 732) | AM279465.1 | (ABT301) | | | AB302791.1 | (IFO0494) | AM943665.1 | (ATCC42981) | AM943655.1 | (CBS 732) |
| | | | | | | | AM911009.1 | (YSF21) | AM911008.1 | (YSF34) | AY524005.1 | (PYCC3693) |
| | | | | | | | AJ783434.1 | (ESAB21) | AJ716118.1 | (CBS9714) | U72163.1 | (Unknown) |
| *Z. mellis* CBS 7412 | FN431890 | | | | | | FN431891 | | | | | |
| | AB363062.1 | (ZR510) | AB363061.1 | (ZR14) | AB363060.1 | (ZR-1) | AB363046.1 | (ZR-1) | AB363042.1 | (SR-4) | AB363041.1 | (SR-2) |
| | AB363057.1 | (SR-4) | AB363056.1 | (SR-2) | AB363055.1 | (KS03) | AB363039.1 | (KS03) | AB363037.1 | (KS01) | AB363036.1 | (KF-4) |
| | AB363054.1 | (KS01) | AB363053.1 | (KF-4) | AB363051.1 | (H14-7-1) | AB363034.1 | (H14-7-1) | AB302813.1 | (IFO 1960) | AB302812.1 | IFO 1914 |
| | AB302834.1 | (IFO 1960) | AB302827.1 | (IFO 1130) | AB302826.1 | (IFO 0845) | AB302809.1 | (IFO 1814) | AB302808.1 | (IFO 1813) | AB302806.1 | (IFO 1130) |
| | AB302825.1 | (IFO 0740) | AB302824.1 | (IFO 0596) | AB302820.1 | (IFO 0513) | AB302804.1 | (IFO 0740) | AB302803.1 | (IFO 0686) | AB302797.1 | IFO0513 |
| | AB302819.1 | (IFO 0512) | AB302818.1 | (IFO 0511) | AM943656.1 | (ATCC 42981) | AB302796.1 | IFO0512 | AB302795.1 | IFO0511 | AB302791.1 | (IFO 0494) |
| | AM943655.1 | (CBS 732) | AM279465.1 | (ABT301) | | | AM943656.1 | (ATCC 42981) | AM943655.1 | (CBS 732) | AM911009.1 | (YSF21) |
| | | | | | | | AM911008.1 | (YSF34) | AY524005.1 | (PYCC 3693) | AJ783434.1 | (ESAB21) |

Sequences retrieved from GenBank.
Accession number printed in bold corresponds to sequences obtained in the current study.

the phenotypic identification was not correct. We therefore concluded that these two strains belong to the *Z. rouxii* species (Fig. 3, Table 3).

Two *Z. rouxii* strains (CECT 11923 and CECT 10425) were confirmed as *Z. rouxii* in this study by sequencing. Although the undoubted value of using simple gene sequences (e.g. 26 S rDNA D1/D2) to identify yeasts is recognized, this method has some limitations for the identification of hybrids (James et al., 2005). Moreover, the fact that both strains appear together with the hybrids (NCYC1682, NCYC 3060 and NCYC 3061) identified by James et al. (2005) in a separate cluster (Fig. 3) may indicate that they could also be hybrids. These authors suggest that yeast hybrids may be more abundant than previously thought; in fact, Solieri et al. (2007) recently reported new *Z. rouxii* hybrids. For this reason, further studies are under way in our laboratory to clarify the nature of these strains. Note that this method also permits all the hybrid strains analysed so far to be distinguished, as shown in Tables 2 and 3.

On the other hand, during this study, we have developed a modification of the method previously described by Romero et al. (2005) and Quirós et al. (2006) (Materials and methods). After the spheroplasted treatment, the DNA extraction was done in half the time using DNeasy (see Materials and methods 2.2). Changing the DNA polymerase and the PCR conditions (see Material and methods 2.2 (ii)) made it possible to amplify IGS regions with a size as high as 7000, 6700 or 6000 bp. The amplicon size of the IGS is different in seven out of the nine species of *Zygosaccharomyces* tested (Table 1). For example, a size of 6300 bp corresponds to *Z. bailii* and 5500 bp to *Z. bisporus*. Although this technique is unreliable as a method of identification, in practice relatively few yeast species are responsible for the majority of food spoilage by yeasts (Stratford, 2006) and some specific associations are frequent and often predictable (Fleet, 2006). This generally narrows the candidates for yeast spoilage in a specific food. Typing methodologies are usually applied after the identification process. For example, *Z. mellis* and *Z. rouxii* may easily be identified from colonies by the size of the amplicon of the 5.8S-ITS region and the fragments obtained after digestion with three restriction endonucleases (Barata et al., 2008; Esteve-Zarzoso et al., 1999, 2003, yeast-id.com (CECT)).

In conclusion, the PCR-RFLP analysis of the IGS region of rDNA is a fast and single molecular typing method producing clear and reproducible restriction RFLP patterns. It constitutes a typing method for the *Z. bailii*, *Z. mellis* and *Z. rouxii* species as well as for other species belonging to the *Zygosaccharomyces* genus. The method also discriminates hybrid strains of *Zygosaccharomyces*. It does not require sequencing technologies and as a consequence is easier to implant in the routine of an industry laboratory.

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# Capítulo 3

***Zygosaccharomyces rouxii* CECT 11923 and *Z. rouxii* CECT 10425: two new putative *Zygosaccharomyces* sp. hybrid strains.** Wrent *et al.* 2015. International Journal of Food Microbiology (en proceso de revisión)

# *Zygosaccharomyces rouxii* CECT 11923 and *Z. rouxii* CECT 10425: two new putative *Zygosaccharomyces* sp. hybrid strains

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**Key words:** Hybrid-specific primers, oriental-fermented-food-products, *Zygosaccharomyces*, *SOD2*, *HIS3*, 5.8S-ITS rDNA polymorphism

**Highlights:**

- Strain CECT 11923 is a hybrid species between *Z.rouxii* and *Z. sapae*
- Strain CECT 10425 might be a hybrid species between *Z. pseudorouxii* and *Z. sapae*
- Both strains differ in *SOD2* and *HIS3* gene copies and present divergent 5.8S-ITS rDNA
- Species–specific primers reveal differences between both strains
- Based on IGS1 polymorphism, we develop hybrid-specific primers

**Abstract**

Based on IGS-PCR RFLP polymorphism, we previously detected two *Z. rouxii* strains (CECT 11932 and CECT 10425) that clustered with hybrid strains (NCYC1682, NCYC3060 and NCYC1682). In the present study, we applied molecular tools to highlight the nature of these strains. Based on the results of D1/D2 26S rDNA sequencing, the sequences of divergent copies of 5.8S-ITS rDNA and the number of copies of nuclear-encoded genes (*SOD2* and *HIS3*), we hypothesize that the CECT 11923 strain is a hybrid species between *Z. rouxii* and *Z. sapae*, whereas CECT 10425 might be a hybrid species between *Z. pseudorouxii* and *Z. sapae.* Given the recently recognized important industrial role of hybrids, their detection is very useful and avoids misinterpretation caused by results due to one or both parental species. Although it was not one of the study objectives, we also developed a pair of hybrid-specific primers, HibZF/HibZR, based on the polymorphism of the IGS1-rDNA region. Positive amplicons were obtained only in the *Zygosaccharomyces* sp. hybrids included in this study and the CECT 11923 and CECT 10425 strains analyzed here.

**1 Introduction**

Yeast belonging to the genus *Zygosacharomyces* colonizes selective food-grade niches, including sugary and salty foods and beverages (Dakal *et al*., 2014). Although they are better known as food spoilage agents when present in foods and beverages, some species of the genus have considerable industrial importance (Sá-Correia *et al*., 2014). On the basis of its halotolerance and production of flavoring compounds, *Z. rouxii* is often introduced as a starter culture (James and Stratford, 2011; Sujaya *et al*., 2003). Together with *Aspergillus oryzae* and *A. sojae*, *Z. rouxii* is

involved in the early fermentation of oriental foods, contributing to their organoleptic properties (Tanaka *et al*., 2012). Examples include miso, a very characteristic product of Japanese cuisine made by fermenting soybeans, and soy sauce, today an ingredient used the world over, which is also produced through the fermentation of soybeans. Recently, it has been reported that putative or confirmed hybrids of *Zygosaccharomyces* play an important role in soy sauce and miso (Suezawa *et al*., 2008; Sujaya *et al*., 2003; Tanaka *et al*., 2012), and artificial hybrids have even been generated in an attempt to improve the flavor characteristics of the products (van der Sluis *et al*., 2001) . Accurate identification is highly desirable from several points of view: first, Type Culture Collection strains are used as a reference in genetic and other studies. Second, polyploidization could optimize the ability to withstand harsh environments (Gordon and Wolfe, 2008) in foods such as industrial miso or balsamic vinegar. It is possible that hybrids exist among the isolated strains identified as *Z. rouxii*, whose physiological properties differ from those of their ancestors. Recently, we developed a method for strain typing Zy*gosaccharomyces* species based on IGS-PCR RFLP (Wrent *et al*., 2010). This revealed important differences between two Z*. rouxii* strains (CECT 11923 and CECT 10425) and the other strains studied. Moreover, these species appeared in a separate cluster together with the natural *Zygosaccharomyces* hybrids identified by James *et al*. (2005) (NCYC1682, NCYC 3060 and NCYC 3061). Here, we elucidate the nature of these strains.

## 2 Materials and methods

### *2.1 Strain and culture conditions*

In this study, we used the *Z. rouxii* CECT 10425 and CECT 11923 strains, as well as 37 other strains for the purposes of comparison. These latter belonged to different species of the *Zygosaccharomyces* genus retrieved from a Type Culture Collection or isolated in our laboratory (Table 1), and included *Zygosaccharomyces* hybrid strains NCYC 1682, NCYC 3060, NCYC 3061 and ATCC 42981 as well as the strain NCYC 3042 provisionally identified as Z. *pseudorouxii.* Similarly, a strain of *Saccharomyces cerevisiae* was also included for comparison purposes. In addition, three species were used for polygenetic analysis as outgroups. Details of culture and maintenance of strains are described elsewhere (Wrent *et al*., 2010).

### *2. 2 DNA isolation and PCR amplification*

DNA was isolated by using the DNeasy plant minikit (Qiagen, Hilden, Germany) after obtaining spheroplasted cells (Quirós *et al*., 2006; Romero *et al*., 2005) and modified by Wrent *et al*. (2010). PCR amplifications were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Germany) and each strain was assayed at least twice.

#### *2.2.1 IGS1 - rDNA Amplification and cloning*

The putative IGS1 homolog region (1078 bp) present in *Z. rouxii* CECT $1232^{T}$ chromosome E was used as amplification target. The sequences were obtained from NCBI (accession number: CU928181), and a pair of primers was designed (Table 2) in order to amplify this region: KONF was selected from the conserved gene 26S-rDNA and the reverse primer KONR from 5S-rDNA. Hairpin formation, 3´complemetarity and potential self-annealing sites were tested by Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). The

primers used were prepared by Laboratorios Conda, Spain. After optimization of PCR conditions, the amplification was carried out as follows; initial denaturalization at 94ºC for 85 sec; 35 cycles at 95ºC for 35 sec, at 58ºC for 55 sec, at 72ºC for 2 min, and then 1 cycle of 72ºC for 10 min. To analyze possible polymorphism, PCR-amplified DNA fragments were separated in 1% (w/v) agarose gel (Bio-Rad) stained with 0.05% (v/v) ethidium bromide (Bio-Rad) and visualized under UV light. The GeneRuler 1kb DNA Ladder (MBI Fermentas) was used as a molecular size marker. Bands were excised from the gel and subsequently purified before cloning using the QIAquick Gel Extraction Kit (QIAgen, Spain). The PCR products of the IGS1- rDNA gene were ligated into the pGEM®-T Easy Vector System (Promega, USA) following the manufacturer's protocol. The ligation products were transformed into *E. coli* JM109 and plated onto Lysogeny Broth (LB) plates containing ampicillin (100 µg mL−1), IPTG (0.1 mM) and X-Gal (200 µg mL−1). To confirm the presence of inserts, white colonies were picked and plasmid DNA was extracted with the NucleoSpin® Plasmid Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. To liberate the fragment, the plasmids were digested with the endonuclease enzyme *BstZ*1 at 50ºC for 2 hours and thereafter separated in agarose gel as mentioned above. Positive IGS1-rDNA inserts presented a size between 1200bp and 1600bp depending on each strain.

*2.2.2 Hybrid-specific primer design*

Based on the alignment of hybrid strains assayed in this study and the *Z.rouxii* CECT 1232$^{T}$ partial IGS1-rDNA region, the HibZ F/R hybrid-specific primers were developed. PCR conditions were 94ºC for 5 min and 26 x (94ºC for 45 s, 59ºC for 1 min and 72ºC for 45 s) with a final extension step of 72ºC for 7 min.

### *2.2.3 Amplification of D1/D2 26S rDNA*

The D1/D2 domains of 26S rDNA were amplified using primers NL1 and NL4 (Kurtzman and Robnett, 1998; O'Donnell, 1993) (Table 2).

**Table 1** Strains used in this work and results obtained with the hybrid- specific primers HibZF/R

| Species | Strain | Origin | HibZF/R (bp) |
|---|---|---|---|
| *Zygosaccharomyces bailii* | CECT 11043 | Cloudy wine | - |
| *Zygosaccharomyces* hybrid | NCYC 3060 | Soya sauce | 700 |
| | NCYC 3061 | Soya sauce | 700 |
| *Zygosaccharomyces kombuachensis* | CBS 8849 | Dried tea fungus | - |
| *Zygosaccharomyces mellis* | CBS 7277 | Alpechin | - |
| | CBS 735 | Fermenting honey | - |
| *Zygosaccharomyces rouxii* | ATCC 42981[a] | Salty miso paste | 750 |
| | CECT 1232[T] | Concentrated must of black grape | - |
| | CECT 1229 | Cane sugar | - |
| | CECT 1231 | Bonbon of bitter-orange syrup | - |
| | CECT 10312 | Unknown | - |
| | CECT 10377 | *Phoenix dactilifera* | - |
| | CECT 10381 | Sugar cane melasse | - |
| | CECT 10425 | Honey | 550 |
| | CECT 10427 | Honey | - |
| | CECT 10445 | Fermenting plum jam | - |
| | CECT 10633 | Honey | - |
| | CECT 11121 | Concentrated grape must | - |
| | CECT 11189 | White wine | - |
| | CECT 11923 | Soya sauce | 750 |
| | CECT 12004 | Cherries in syrup, fermented | - |
| | NCYC 1682[b] | Salty miso paste | 700 |
| | CYC 1484 | Unknown | - |
| | CYC 1486 | Honey | - |
| | CYC 1487 | Nougate | - |
| | T2R | Nougate | - |
| | Bch | Chocolate cake | - |
| | TY1.1 | Nougate | - |
| | TYN1.3 | Nougate | - |
| | TYN2.1 | Nougate | - |
| | N2 | Nougate | - |
| | N3 | Nougate | - |
| *Zygosaccharomyces pseudorouxii* | NCYC 3042 | UK soft drinks factory | - |
| *Zygosaccharomyces sapae* | ABT 301[T] | Balsamic vinegar | - |
| *Saccharomyces cerevisiae* | ATCC 7754 | Fleischmann bakers yeast | - |

[a] Proposed as a hybrid species by Gordon and Wolf, 2008

[b] Proposed as a hybrid species by James *et al* .,2005

**Table 2** Primers used in this work

| Primer pair | Forward 5´--3´ | Reverse 5´--3´ |
|---|---|---|
| **KONF/KONR** | **CGGTAAGAGACTCAGAGAGTA** | **CTGAACGCCTCTAAGTCAGAA** |
| **HibZF/HibZR** | **GATACACGCAGAGCAGATGG** | **TTAGCCAAAGACAGGAGCAGA** |
| NL1/NL4 [a] | GCATAT CAATAAGCGGAGGAAAAG | GGTCCGTGTTTCAAGACGG |
| *Z. bailii* [b] | TATTGATGACCACCACACCACTGAG | TAGACAAGTCAACGACAGCACGCC |
| *Z.bisporus* [b] | GAGGTTCCAGAGTCAATTTTGGG | TAGATCTATAACAGCGTAGGGTCTGTTG |
| *Z. kombuchaensis* [b] | CAAAAGGATCACGAATGAGACTAAG | GCCCAAGTTGATCACTGCGTAAG |
| *Z. lentus* [b] | CACAGGCCACTGGCTCTCAAGTG | ACCTAAGTCAATAACTGCGTATG |
| *Z. mellis* [b] | GGTTGAGAGCGATGGTGTAGCCACA | TGCACCGTCCCAGGCCTTGTC |
| *Z.pseudorouxii* [b] | GTAACGGTGTAGCCACACAG | AGGTTGCCTCTCTGAGAGCC |
| *Z. rouxii* [b] | AGGTAAGAAGAGAGTTGAAAGT | TGCTTCAGTAAAACTTTCTAGA |
| SODIF/SODIR[c] | ACGTATGAATTCGATGCAGAT | TCCTTAACAAACAATGCTAAGT |
| SOD2F/SOD2R[c] | TACATATCAATCAGATTCTAGTT | TTGAAACGCGATGCTGGTC |
| HIS3F/HIS1R[c] | GATYGAYATTCATACYGGTGTYGG | GAAGTTGCTTCTCTAAGGGCT |
| HIS2F/HIS2R[c] | GTAACGGTGTAGCCACACAG | AGGTTGCCTCTCTGAGAGCC |
| its1/ its4[d] | TCCGTAGGTGAACCTGCGG | TCCTCCGCTTATTGATATGC |

In bold primers designed in this work

[a] White *et al* ., 1990

[b] Species-specific primers designed by Harrison *et al* ., 2011

[c] James *et al* ., 2005

[d] O´Donnell *et al* ., 1993; Kurtzman and Robnett 1998

### *2.2.4 SOD2 and HIS3 copy number*

In order to investigate the copy number of two nuclear-encoded *SOD2* genes (encoding an $Na^+$/ $H^+$- antiporter) (Watanabe *et al*., 1991) and the *HIS3* gene (encoding imidazoleglycerol-phosphate dehydratase) (Alifano *et al*., 1996), primer sets were used for the strains listed in Table 3. The primers and amplification parameters were as previously described by James *et al*. (2005), namely SODIF/SODIR (CECT $1232^T$ Zr-*SOD2-22* gene), SOD2F/SOD2R (ATCC 42981 *Z-SOD22* gene), HIS3F/HIS1R (CECT $1232^T$ *HIS3* gene) and HIS2F/HIS2R (NCYC 3042 *HIS3* gene).

### *2.2.5 Amplification and Cloning of 5.8 S ITS rDNA PCR products*

The region encompassing the 5.8S rDNA, and the ITS1 and ITS2 regions (5.8S-ITS region) were also amplified in the CECT 11923 and CECT10425 strains using primers its1 and its4 (Table 2) (White *et al*., 1990). PCR-

amplified fragments were excised from the gel and cloned as described for IGS1-rDNA.

*2.3 DNA sequencing and sequence analysis*

In all cases (IGS1 rDNA, 5.8S-ITS rDNA and D1/D2 26S rDNA), PCR products were cleaned using the Ultraclean™ PCR clean-up Kit (MO-BIO, Larsband, USA). PCR products were sequenced by STAB Vida Lda. (Portugal) using an ABI 3730XL sequencer (Applied Biosystems, USA). Sequences were edited with the EditSeq program included in DNAstar 7.1 software (Lasergene, USA). Each set of sequences (IGS1 rDNA, 5.8S-ITS rDNA and D1/D2 26S rDNA) were aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). The edited sequences were compared with those previously available on the NCBI databases using the BLAST program.

Phylogenetic trees were constructed using the free MEGA6 software (Tamura *et al*., 2013). The sequences were analyzed by Maximum Parsimony (MP). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000).

*2.5 Physiological characterization*

Fermentation of 11 compounds, including maltose, was performed according to Kurtzman *et al*. (2011).

## 3 Results

Previously in our laboratory (Wrent *et al*., 2010), polymorphism of the intergenic spacer region (IGS-PCR RFLP) enabled us to detect two *Z. rouxii* strains (CECT 11932 and CECT 10425) presenting patterns that clustered with the hybrid strains (NCYC 1682, NCYC 3060 and NCYC 3061) described by James *et al*. (2005). As the entire IGS region is quite large in *Z. rouxii*

(4200bp), we decided to study intergenic spacer 1 (IGS1-rDNA). Amplification of the IGS1 region showed differences in size between the 6 strains of *Z. rouxii* studied here, the strains CECT 11923 (1300 pb), CECT 10425 (1200 pb), the hybrid strains NCYC 1682, NCYC 3060, NCYC 3061 (1600 pb) and *Z. pseudorouxii* NCYC 3042 (1500 pb) (data not shown). When further exploring this region, we found that IGS1 rDNA presented a large polymorphism between strains and within the different clones of each strain studied (Fig. 1).

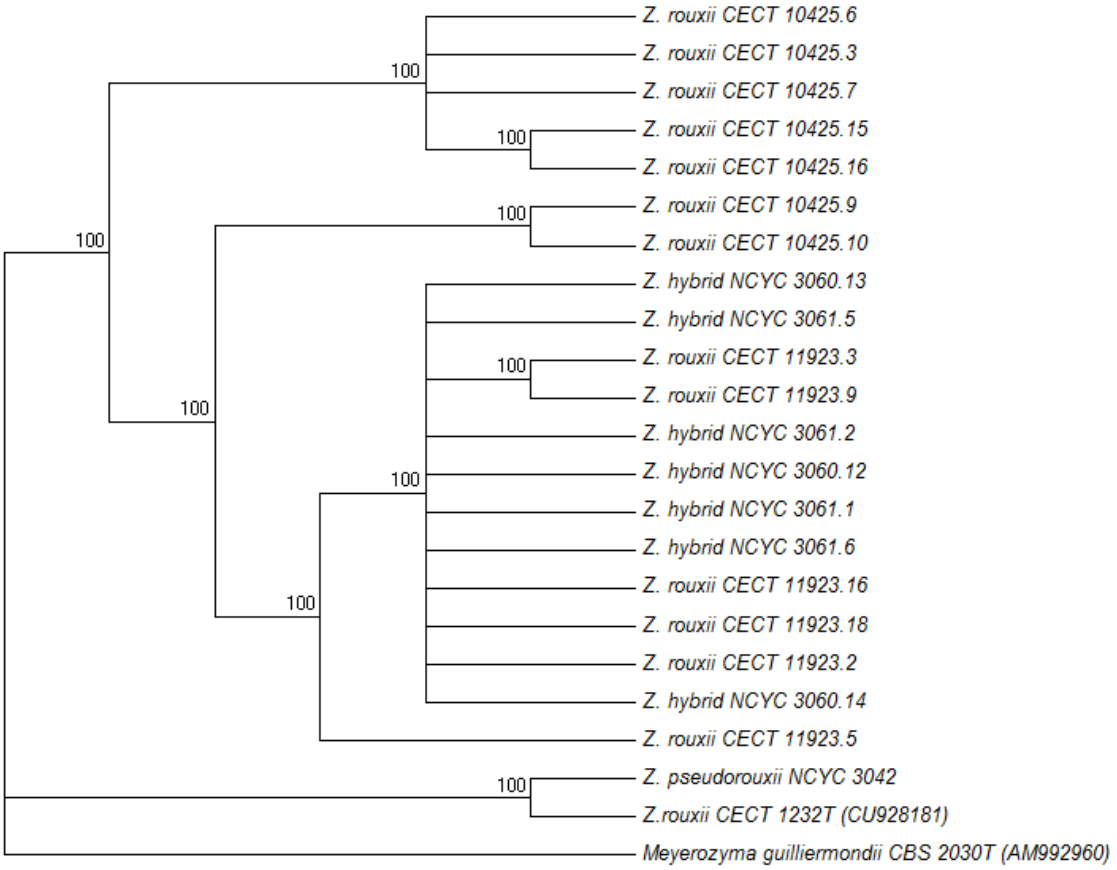


**Fig.1** The Maximum Parsimony tree based on the IGS1 sequences of the *Z. rouxii* CECT 11923, CECT 10425 and CECT 1232$^{T}$, hybrid strains NYCY 3060 and NCYC 3061 clone copies, as well as Z. pseudorouxii NCYC3042. The tree was rooted by inclusion *Meyerozyma guilliermondii* CBS 2030$^{T.}$ Branches corresponding to partitions reproduced in less than 60% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown above the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR). The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated.

There were a total of 953 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

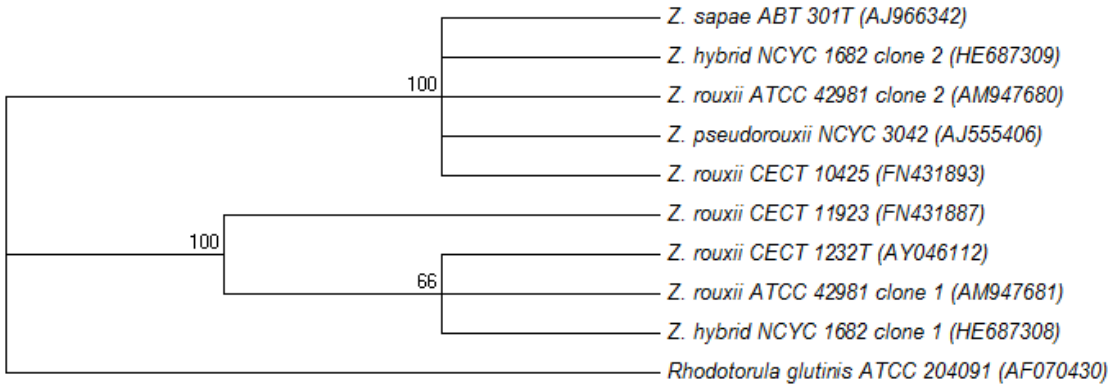


**Fig.2.** The Maximum Parsimony tree based on the 26S rDNA D1/D2 domain sequences of the *Z. rouxii* CECT 11923, CECT 10425 and relatives. The tree was rooted by inclusion *Rhodotorula glutinis* ATCC 204091. Branches corresponding to partitions reproduced in less than 60% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown above the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR). The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 441 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 .

Strain NCYC 3060 presented three different IGS1 types (three clones studied), while we found two different types in strain NCYC 3061 (four clones analyzed). There were three clone types in strain CECT10425, and all the clones analyzed in strain CECT 11923 possessed different types (six clones analyzed). The last 97 nucleotides in the 3´region did not present any polymorphisms between these strains, and neither did the first 37 nucleotides in the 5´region (data not shown). When comparing these two sets of sequences with *Z.rouxii* CECT 1232$^{T}$ and with *Z.pseudorouxii* NCYC 3042, we observed that they were quite different, and these differences enabled us to design hybrid- specific primers (HibZ F/R). Subsequently, their suitability was confirmed by PCR amplification of the yeast strains listed in Table 1.

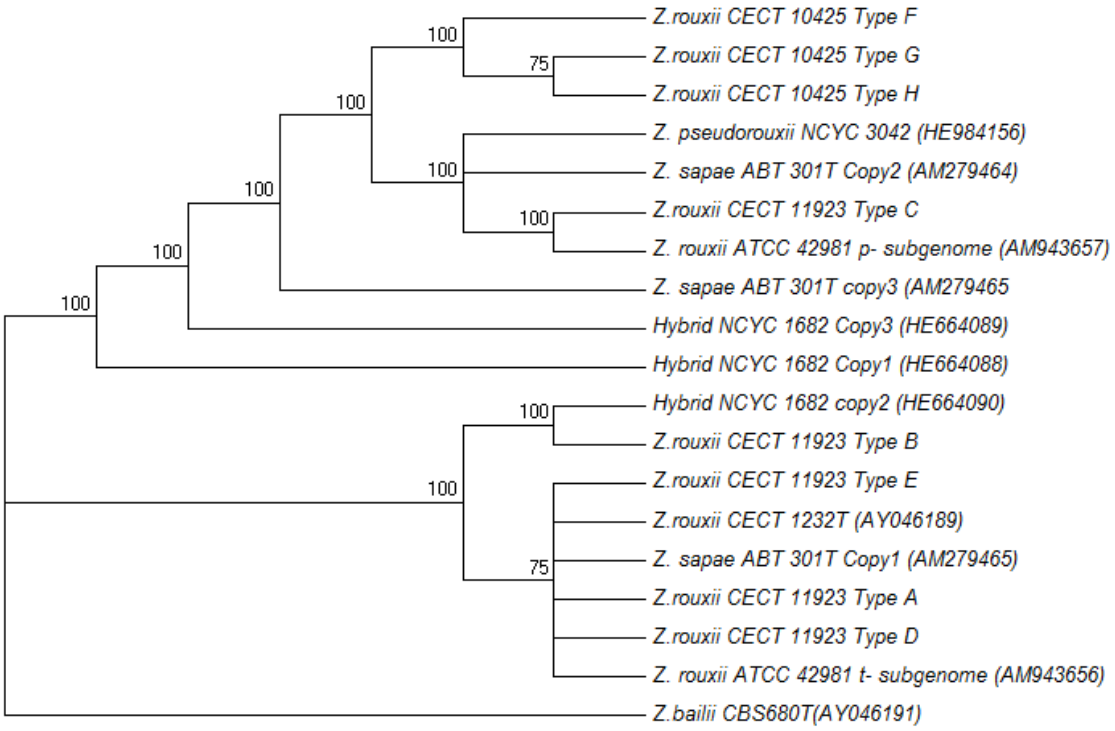


**Fig .3.** The Maximum Parsimony tree based on the 5.8S-ITS rDNA region of the *Z. rouxii* CECT 11923, CECT 10425 clone copies and relatives. The tree was rooted by inclusion *Zygosaccharomyces bailii CBS 680*$^{T}$. Branches corresponding to partitions reproduced in less than 60% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown above the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR). The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 573 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

The HibZ primers amplified all strains described as hybrids by James *et al*. (2005), ATCC 42981 described as a hybrid by Gordon and Wolfe, (2008) and the strains suspected to be hybrids studied here, CECT 11923 and CECT 10425. However, the primers did not amplify the IGS1 rDNA region in *Z. sapae* nor in *Z. pseudorouxii*. A clear single fragment from 700bp to 750bp was obtained with the strains isolated from soy sauce and salty miso paste (CECT 11923, NCYC3060, NCYC3061, NCYC1682, and ATCC 42981), while the strain isolated from honey (CECT10425) presented an amplicon of 550 bp (Table 1).

The taxonomic status of strains CECT 11932 and CECT 10425 was further studied by identification with the genus-specific primers previously described by Harrison *et al*. (2011) (Table 4), amplification of the nuclear SOD2 and *HIS3* genes (Table 3) and additional sequence analysis of the D1/D2 26S rDNA region as well as sequence analysis of 5.8S-ITS rDNA copies (Figs. 2 and 3).

**Table 3** Copy number of *SOD2* and *HIS3* genes

| | | | | *SOD2* | | *HIS3* | |
|---|---|---|---|---|---|---|---|
| *Species* | Strains | Other collectio | Origin | Copy 1 SOD1F/SOD | Copy 2 SOD2F/SOD | Copy 1 HIS3F/HIS | Copy2 HIS2F/HIS2R |
| *Zygosaccharomyces* Hybrids | NCYC 3060 | CBS 9952 | Soya sauce | + | + | + | + |
| | NCYC 3061 | CBS 9953 | Soya sauce | + | + | + | + |
| *Zygosaccharomyces pseudorouxii* | NCYC 3042 | CBS 9951 | UK soft drinks factory (sugar) | - | + | - | + |
| *Zygosaccharomyces rouxii* | CECT 1232$^T$ | CBS 732$^T$ | Concentrated must of black g | + | - | + | - |
| | CECT 1229 | CBS 7804 | Cane sugar | + | - | + | - |
| | CECT 1231 | CBS 686 | Bonbon of bitter-orange syru | + | - | + | - |
| | CECT 10132 | | Unknown | + | - | + | - |
| | CECT 10312 | | Fig cake | + | - | + | - |
| | CECT 10377 | | *Phoenix dactilifera* | + | - | + | - |
| | CECT 10425 | | Honey | - | + | + | + |
| | CECT 10427 | | Honey | + | - | + | - |
| | CECT 10445 | | Fermenting plum jam | + | - | + | - |
| | CECT 10633 | | Honey | + | - | + | - |
| | CECT 11923 | | Soy sauce | + | + | + | + |
| | ATCC 42981 | | Miso | + | + | + | + |
| | NCYC 1682 | CBS4837 | Salty miso paste | + | + | + | + |
| | T2R | | Nougate | + | - | + | - |
| | BcH | | Chocolate cake | + | - | + | - |
| *Zygosaccharomyces sapae* | ABT 301$^T$ | | Balsamic vinegar | + | + | + | + |

**Table 4** Specie-specific primers for His3 amplification desribed by Harrison *et al.* (2011).

| *Species* | Collections | *Z.rouxii* | *Z.lentus* | *Z.bailii* | *Z.bisporus* | *Z.mellis* | *Z.kombuchaensis* | *Z.pseudorouxii* |
|---|---|---|---|---|---|---|---|---|
| *Zygosaccharomyces rouxii* | CECT 1232$^T$ | + | - | - | - | - | - | - |
| | CECT 10425 | - | - | - | - | - | - | + |
| | CECT 11923 | + | - | - | - | - | - | + |
| | NCYC 1682 | + | - | - | - | - | - | + |
| | ATCC 42981 | + | - | - | - | - | - | + |
| *Zygosaccharomyces lentus* | CECT 11041 | - | + | - | - | - | - | - |
| *Zygosaccharomyces bailii* | CECT 11042 | - | - | + | - | - | - | - |
| *Zygosaccharomyces bisporus* | CECT 11348 | - | - | - | + | - | - | - |
| *Zygosaccharomyces mellis* | CBS 1091 | - | - | - | - | + | - | - |
| *Zygosaccharomyces kombuchaensis* | CBS 8849 | - | - | - | - | - | + | - |
| *Zygosaccharomyces* hybrids | NCYC 3060 | + | - | - | - | - | - | + |
| | NCYC 3061 | + | - | - | - | - | - | + |
| *Zygosaccharomyces pseudorouxii* | NCYC 3042 | - | - | - | - | - | - | + |
| *Zygosaccharomyces sapae* | ABT 301$^T$ | + | - | - | - | - | - | + |

Unlike strain CECT 10425 (Table 4), identification of the strain CECT 11923 with genus-specific primers produced two amplicons that corresponded to *Z. rouxii* and *Z. pseudorouxii.* This also occurred in all hybrid strains tested

(NCYC 1682, NCYC 3060 and NCYC 3061) as well as in *Z. sapae* ABT 301$^T$ and in *Z. rouxii* ATCC 42981.

Regarding the nuclear-encoded *SOD2* and *HIS*3 genes, the CECT 10425 strain possessed one copy of the *SOD2* gene, like *Z. pseudorouxii*, but two copies of *HIS* 3, unlike *Z. pseudorouxii,* whereas the CECT 11923 strain possessed two copies of both genes.

With respect to the analysis of sequences, when the D1/D2 26S rDNA region was BLASTed, the CECT 10425 strain (accession number: FN431893) presented a sequence which was identical to *Z. pseudorouxii* (accession number: AJ555406) and Z. *sapae* ABT 301$^T$ (accession number: AJ966342) and differed by 1 base substitution from the *Z. rouxii* NCYC 1682 clone2 (accession number: HE687309) and by 12 bases from the *Z. rouxii* type strain (CECT 1232$^T$*).* In contrast, strain CECT 11923 presented a sequence identical to the type strain of *Z. rouxii* and differed only by one base from clone 1 of *Z. rouxii* NCYC 1682 as well as from clone 1 of ATCC 42981. Fig.2 shows the dendrogram.

**Table 5** BLASTed gene copies of 5.8s-ITS rDNA of strains *Zygosaccharomyces rouxii* CECT 10425 and CECT 11923

| Clones (numbers) | Type | Homology (%) | Species | Strain | Homology (%) with *Z. sapae* strain M21[a] |
|---|---|---|---|---|---|
| 11923 (2) | A | 99 | *Z. rouxii* | CECT1232$^T$ | 97 Copy2 and 80 Copy1 |
| 11923 (1) | B | 97 | *Z. rouxii* | NBRC 10668[b] | 89 Copy2 and 92 Copy1 |
| 11923 (1) | C | 97 | *Z. rouxii* | ATCC 42981 | 96 Copy2 and 79 Copy1 |
| 11923 (7) | D | 100 | *Z. rouxii* | CECT1232$^T$ | 98 Copy2 and 80 Copy1 |
| 11923 (1) | E | 99 | *Z. rouxii* | NBRC 10672[b] | 96 Copy2 and 79 Copy1 |
| 10425 (2) | F | 99 | *Z. rouxii* | NBRC 10669 | 98 Copy2 and 80 Copy1 |
| 10425 (3) | G | 99 | *Z. rouxii* | NBRC 10669 | 98 Copy2 and 80 Copy1 |
| 10425 (3) | H | 99 | *Z. rouxii* | NBRC 10669 | 98 Copy2 and 82 Copy1 |

[a]Clones BLASTed only against *Z.sapae* strain

[b]This strain was formarly identified as *Z. rouxii* . On the basis of the LSUrDNA D1/D2 sequences, this strain may be considerd conspecific with *Z. sapae* .

Table 5 lists the different copies obtained for the 5.8S-ITS rDNA region. As can be seen, 5 different copies were found for strain CECT 11923 (arbitrarily denominated as A to E) and 3 (F, G, H) for strain CECT 10425. The 5.8S-ITS rDNA sequence copy D (CECT 11923) presented 100% sequence identity with the *Z. rouxii* type strain, and copy A was also closely related (99% sequence identity). Meanwhile, the 5.8S-ITS rDNA sequence copies B and E were closer (97% and 89%, respectively) to strain *Z. rouxii* NBCR 10668, considered conspecific with *Z. sapae* and *Z. sapae* ABT 301$^{T}$. The three copies of 5.8S-ITS found in the CECT 10425 strain were more similar to each other, with the highest difference being between F and H (one base substitution and one gap), and all of them presented 99% homology with strain *Z. rouxii* NBCR 10669. The phylogenetic tree constructed for the different 5.8S-ITS rDNA sequences represented in Fig. 3 clustered the CECT 11923 non-orthologous 5.8S-ITS rDNA sequences in various groups, while all copy types found in CECT 10425 were clustered in the same group.

Lastly, the strains CECT 10425 and 11923 differed from the type strain *of Z. rouxii* CECT1232$^{T}$, hybrid strains NCYC 3060 and NCYC 3061 and *Z. pseudorouxii* NCYC 3042 in their inability to ferment maltose, like *Z. sapae* ABT 301$^{T}$ (data not shown).

## 4 Discussion

The taxonomic status of *Zygosaccharomyces* sp. has always been controversial (Sá-Correia *et al*., 2014). Currently, in the latest edition of *The Yeasts* (Kurtzman *et al*., 2011), the genus *Zygosaccharomyces* was reduced to six species (Z*. bailii*, *Z. bisporus*, *Z. kombuachensis*, *Z. lentus*, *Z. mellis* and *Z. rouxii*).

Hybridization between the same or two species occurs occasionally in nature, and the resulting hybrids are sometimes viable and can propagate (Groth *et al*., 1999). Hybridization processes are suspected to have played a role in species evolution (Wu *et al*., 2008). The increased genome size and complexity of hybrids may sometimes result in an adaptative advantage thanks to greater stress resistance or fecundity (Piotrowski *et al*., 2012). Natural hybrid strains of *Saccharomyces* isolated from wine or beer are perhaps the most compelling examples of this (Borsting *et al*., 1997; Casaregola *et al*., 2001; de Barros Lopes *et al*., 2002; Nguyen *et al*., 2000; Peris *et al*., 2012). Several artificial hybrids of *S. cerevisiae* have even been generated purely to determine their hybrid characteristics (Bellon *et al*., 2011; Sipiczki, 2008). As with *Saccharomyces*, it has been reported that *Z. rouxii* is capable of producing stable natural inter-specific hybrids (James and Stratford, 2011).

As mentioned in the Introduction, in a previous study carried out in our laboratory (Wrent *et al*., 2010), we developed a strain typing method for *Zygosaccharomyces* yeast species based on the polymorphism of the intergenic spacer region (IGS). By means of this method, we found that two *Z. rouxii* strains (CECT 11932 and CECT 10425) presented a similar pattern to those of the hybrids (NCYC 1682, NCYC 3060 and NCYC 3061). Thus, we hypothesized that this might indicate that they were also hybrids. The CECT 11923 strain (CBS 4021) was isolated from soy sauce by Onishi and deposited in CBS as *S. acidifaciens* var. *halomembranis*, although it was later reclassified by Kurtzman as *Z. rouxii* (data from CBS), and CECT 10425 was isolated and identified by Santa- María from honey and deposited in CECT in 1991 (data from CECT). Given the now recognized important industrial role of hybrids, their detection is very

useful and avoids misinterpretation caused by results due to one or both parental species. With this objective, Solieri *et al*. (2015) have developed methods for identifying inter- and intra-species *Saccharomyces* hybrids. Based on the polymorphism found in the IGS1-rDNA region, here we have developed a method that makes it possible to recognize hybrid strains of *Zygosaccharomyces* sp. (Table 1). The primers HibZF/ HibZR developed in this study produced clear fragments in all of the hybrid strains assayed (ATCC 42981, NCYC 1682, NCYC 3060 and NCYC 3061), as well as in the strains CECT 11923 and CECT 10425. As expected, due to the differences found in the IGS1-rDNA sequence, the primers did not amplify the novel species *Z. pseudorouxii* nom. inval., *Z. rouxii* or *Z. sapae* ABT 301$^{T}$(Table 1). In accordance with Solieri *et al*. (2013), this latter result might confirm that although *Z. sapae* resembles a hybrid strain, in fact it is not. It is notable that this method also recognized the allodiploid ATCC 42981 strain, which has a p-subgenome, supposedly retrieved from Z. *pseudorouxii*, and a t-subgenome identical to *Z. rouxii* (Gordon and Wolfe, 2008; James *et al*., 2005; Solieri *et al*., 2013; Suezawa *et al*., 2008). In fact, this strain is considered an interspecies hybrid that was formed so recently that its genome has not yet had time to decay (Gordon and Wolfe, 2008). On the other hand, no false positives were found in the other *Zygosaccharomyces* species assayed, or in *S. cerevisiae* (Table 1). Data from this study suggest that the CECT 11923 strain could be a hybrid strain because it possess two copies of *SOD*2 and *HIS* 3 (Table 3), it was recognized by specific primers for *Z. rouxii* and *Z. pseudorouxii*, and one of the ITS copies (a copy arbitrarily named D) presented 100% homology with the *Z. rouxii* type strain and the others with strains recently described as being conspecific with *Z.sapae.* On the other hand, like *Z. sapae* but in

contrast to *Z. rouxii* and *Z. pseudorouxii*, CECT 11923 does not ferment maltose.

With respect to strain CECT 10425, the results obtained from species-specific primers (Harrison *et al*., 2011) (Table 4), together with the D1/D2 domain sequence results (Fig.2) (100% homology with *Z.pseudorouxii* NCYC 3032*, Z. sapae* ABT 301 $^{T}$ and *Z. rouxii* ATCC 42981) suggest that the CECT 10425 strain could be considered a new strain of *Z. pseudorouxii* or *Z. sapae*. These results are not surprising since *Z. pseudorouxii and Z. sapae* are similar. *Z. pseudorouxii* NCYC 3042 has not been formally described and is considered an ancestor of *Z. sapae.* Solieri *et al*. (2013) found that although *Z. pseudorouxii* NCYC 3042 and *Z. sapae* had an identical D1/D2 26S rDNA sequence, physiological and genetic studies revealed that they presented marked differences. However, we hypothesize that the CECT 10425 strain may also be a hybrid species, based on the results obtained previously (Wrent *et al*., 2010), analysis of IGS sequences (Fig. 1), the results obtained with the hybrid-specific primer designed in this study (Table 1), the number of divergent copies of *SOD2* and *HIS3* (namely, the *HIS3* gene from *Z.rouxii* and the *HIS3* gene from *Z. pseudorouxii* NCYC 3042), the fact that it only possesses the ATCC 42981 *Z-SOD22* gene, the absence of maltose fermentation, like *Z. sapae,* and the fact that strain CECT 10425 possesses three ITS-5.8-rDNA copies of which all presented 99% homology with the *Z. rouxii* NBRC 10669 strain conspecific with *Z. sapae*.

In conclusion, strain CECT 11923 could be a hybrid strain between *Z. rouxii* and *Z. sapae,* and CECT 10425 could be a hybrid between *Z. pseudorouxii* and *Z.sapae*. Although it was not one of the study objectives, the IGS1 differences found between hybrids and non-hybrids enabled us to design

a pair of hybrid-specific primers. Both strains as well as other hybrids (Gordon and Wolfe, 2008; James *et al*., 2005) gave positive results with the hybrid-specific primers, which constitute a rapid and affordable method for the detection of *Zygosaccharomyces* hybrid species.

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# Capítulo 4

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# Development of species-specific primers for rapid identification of *Debaryomyces hansenii*

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ABSTRACT

In this work, we developed a specific PCR assay for *Debaryomyces hansenii* strains that uses a putative homologous *PAD1* region (729 bp) present in this yeast species as a target. The amplification of this sequence with the *D. hansenii* specific primer pair (DhPADF/DhPADR) was found to be a rapid, specific and an affordable method enabling identification of *D. hansenii* from other yeast strains. Primers were tested in almost 100 strains, 49 strains from Type Culture Collection belonging to the genus *Debaryomyces* and to other yeast species commonly found in foods or related genera. These primers were able to discriminate between closely related species of *Debaryomyces*, such as *Debaryomyces fabryi* and *Debaryomyces subglobosus*, with a 100% detection rate for *D. hansenii*. Also, the method was tested in 45 strains from different foods. Results confirmed the specificity of the PCR method and detected two earlier misidentifications of *D. hansenii strains* obtained by RFLP analysis of the 5.8S ITS rDNA region. Subsequently we confirmed by sequencing the D1/D2 domain of 26S rDNA that these strains belonged to *D. fabryi*. We call attention in this work to the fact that the RFLPs of the 5.8S ITS rDNA profiles of *D. hansenii*, *D. fabryi* and *D. subglobosus* are the same and this technique will thus lead to incorrect identifications.



## 1. Introduction

The yeast species *Debaryomyces hansenii* is widespread in nature (Kurtzman et al., 2011) and has been extensively studied because of its halotolerance and potential industrial applications (Wrent et al., 2014). In fact, it is one of the most frequently isolated yeasts among secondary microorganisms involved in the ripening process of traditional cheese (Dolci et al., 2009), contributing to distinctive organoleptic characteristics (Padilla et al., 2014; Petersen et al., 2002). Likewise, it is an important contribution to flavor development in traditional dry-cured meat products such as sausages or ham as has been reported (Cano-García et al., 2014; Martín et al., 2006). In addition, *D. hansenii* seems to be suitable as a biocontrol agent for reducing risk caused by mycotoxin producing molds in dry-cured ham, dairy products and fruits (Andrade et al., 2014; Hernandez-Montiel et al., 2010; Liu and Tsao, 2009).

For laboratories and industries an accurate, quick and affordable identification is very useful. Yeast identification based on morphological and physiological criteria is nowadays little used because it is much slower and less accurate than molecular techniques. Based on genetic differences previously reported (Corredor et al., 2000; Groenewald et al., 2008; Prillinger et al., 1999; Quirós et al., 2006), strains that formerly were included in *D. hansenii* have been recently reinstated as new species of the genus, i.e. *Debaryomyces fabryi*, *Debaryomyces subglobosus* (Kurtzman et al., 2011). They show close physiological similarity to *D. hansenii*, resulting in numerous misidentifications.

Identification by sequence analysis is expensive and time consuming when it comes to large scale work, and can take days if the sequencing facilities are outside the workplace (Hulin and Wheals, 2014). Gente et al. (2007) developed primer-pairs that are able to discriminate between a large number of different yeast species directly from the surface of smear-ripened cheese, among them *D. hansenii*, but there was no reproducible DNA amplification with the strain *D. hansenii* CBS 766 (one of the three assayed). Other methods such as RAPD and mtDNA have been proposed as an alternative to traditional characterization at species- and at intra-species levels (Nikolaou et al., 2007; Querol et al., 1992). However, Andrade et al. (2010) concluded that RAPD was not able to discriminate between species such as *D. hansenii* and *Candida zeylanoides* and though mtDNA was more reliable, and some of the isolates could be characterized directly as belonging to *D. hansenii* species, it failed to identify the majority. Corredor et al. (2000) developed two probes for *D. hansenii* var *hansenii*, but this

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**Table 1**
Yeast strains and PCR products obtained with the DhPadF/DhPadR pair of primers.

| Species | Strains | Other collections | Origin | Amplification with DhPadF/DhPadR (400 bp) |
|---|---|---|---|---|
| *Candida cretensis* | CECT 12038 | | Pork sausage | − |
| *Debaryomyces fabryi* | CBS 6066 | ATCC 22262 | Tanning fluid | − |
| | CECT 11365 | CBS 4373 | Dry white wine | − |
| | CECT 11370[T] | ATCC 20278; CBS 789 | Interdigital mycotic lesion | − |
| *Debaryomyces hansenii* | CBS 116 | | Cherries | + |
| | CBS 164 | | Cheese | + |
| | CBS 766 | ATCC 10623 | Cheese | + |
| | CBS 1102 | CECT 11364 | Beef-and-pork sausage | + |
| | CBS 1792 | CECT 11363 | Chilled beef | + |
| | PYCC 4745 | | Sea water | + |
| | CECT 10019 | | Frass on *Philadelphus coronarius* | + |
| | CECT 10026 | | Salt cod | + |
| | CECT 10038 | | Frass on *Alnus glutinosa* | + |
| | CECT 10517 | | Alpechin | + |
| | CECT 10284 | | Frass on *Cornus sanguinea* | + |
| | CECT 10352 | | Tomato | + |
| | CECT 10353 | | Tomato | + |
| | CECT 10360 | | Cheese | + |
| | CECT 10378 | | Date | + |
| | CECT 10386 | | Prune | + |
| | CECT 10414 | | Alpechin | + |
| | CECT 11369[T] | NRRL Y-7426[T]; CBS 767[T] | Carlsberg Laboratories | + |
| *Debaryomyces maramus* | CBS 4264 | CECT 11371 | Cider | − |
| | CECT 11362[T] | ATCC 11627[T]; CBS 1958[T] | Atmosphere | − |
| *Debaryomyces prosopidis* | CBS 8450[T] | ATCC 201611[T] | Exudate of *Prosopis juliflora* (mesquite tree) | − |
| | DBVPG 7012 | ATCC MYA-255 | Exudate of mesquite trees | − |
| *Debaryomyces subglobosus* | CBS 792[T] | JCM 1989[T] | Infected nail | − |
| | CBS 1796 | | Skin lesion | − |
| *Debaryomyces udenii* | CBS 7057 | JCM 7856 | Soil | − |
| *Hanseniaspora uvarum* | CECT 10142 | | Cucumber | − |
| | CECT 11105 | CBS 2589 | Grape must | − |
| *Kluyveromyces lactis* | CECT 1132 | ATCC 48432 | Brine | − |
| | CECT 10361 | | Cheese | − |
| *Meyerozyma guilliermondii* | CECT 1456[T] | ATCC 46036[T]; CBS 2030[T] | Insect frass on *Ulmus americana* (elm tree) | − |
| *Priceomyces carsonii*[a] | CECT 10227[T] | ATCC 58371[T]; CBS 2285[T] | Slime flux of *Quercus kelloggii* (black oak) | − |
| *Rhodotorula glutinis* | CECT 10145[T] | ATCC 96365[T]; CBS 2367[T] | Fruit of *Phyllodendron* sp. | − |
| *Saccharomyces cerevisiae* | ATCC 7754 | CBS 1368 | Fleischmann bakers yeast | − |
| *Schwanniomyces pseudopolymorphus*[b] | CECT 11360[T] | ATCC 24211[T]; CBS 2008[T] | Tanning fluid, prepared from bark of sweet-chestnut | − |
| *Schwanniomyces yamadae*[c] | CBS 7035[T] | ATCC 56471[T];CECT 11416[T] | Soil of grassland | − |
| *Torulaspora delbrueckii* | ATCC 66821[T] | CBS 1146[T]; CECT 11199[T] | Unknown | − |
| *Wickerhamomyces anomalus*[d] | CECT 1114[T] | ATCC 8168[T]; CBS 5759[T] | Unknown | − |
| | CECT 12806 | | Grape juice | − |
| *Yarrowia lipolytica* | CECT 10363 | | Butter | − |
| *Zygosaccharomyces bailii* | CECT 1898[T] | ATCC 58445[T]; CBS 680[T] | Brewery | − |
| *Zygosaccharomyces cidrii*[e] | CECT 10657[T] | ATCC 36238[T]; CBS 4575[T] | Cider | − |
| *Zygosaccharomyces fermentati*[f] | CECT 11056[T] | ATCC 58446[T]; CBS 707[T] | Sediment of peppermint beverage | − |
| *Zygosaccharomyces mellis* | CBS 1091 | DBVPG 6460 | Fermenting honey | − |
| | CECT 10127 | | Honey | − |
| *Zygosaccharomyces rouxii* | CECT 1232[T] | ATCC 2623[T]; CBS 732[T] | Concentrated must of black grape | − |

ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; CECT: Colección Española de Cultivos Tipo; DBVPG:Industrial Yeasts Collection; JCM: Japan Collection of Microorganisms.
PYCC: Portuguese Yeast Culture Collection.
[T] Type strain.
[a] Formerly *Debaryomyces carsonii* (Kurtzman and Fell, 1998).
[b] Formerly *Debaryomyces pseudopolymorphus* (Kurtzman and Fell, 1998).
[c] Formerly *Debaryomyces yamadae* (Kurtzman and Fell, 1998).
[d] Formerly *Pichia anomala* (Kurtzman and Fell, 1998).
[e] Now *Lachancea cidri* (Kurtzman et al., 2011).
[f] Now *Lachancea fermentati* (Kurtzman et al., 2011).

method requires a Dot blot analysis of DNA to recognize the yeast species that hybridize with the probes, which makes it more expensive and slower than our proposal.

The *PAD1* gene in *Saccharomyces cerevisiae* encodes for phenylacrylic acid decarboxylase which confers resistance to cinnamic acid (Clausen et al., 1994). Other putative *PAD1* homologues have been found in yeast species such as *Candida albicans*, *Candida dubliniensis*, *D. hansenii and Wickerhamomyces anomalus*. All of them presented decarboxylation of sorbic acid (Stratford et al., 2007). During a study carried out in our laboratories on decarboxylation of sorbic acid in *D. hansenii* we observed that the type strain presented a putative homologous region of the *PAD1* gene of *S. cerevisiae*. As significant differences in the homologous regions of the two were found, we thought that this sequence could be a good target for the *D. hansenii* specific detection and extended the comparison to other *D. hansenii* strains and yeast species.

The aim of the present study was to develop a specific primer for a rapid and affordable identification of the yeast species *D. hansenii* based on this region.

**Table 2**
Yeast strains and PCR products obtained with the DhPadF/DhPadR pair of primers.

| Strains | | Isolation source | Amplification with DhPadF/DhPadR |
|---|---|---|---|
| *Candida zeylanoides* | Ca2[a] | Spanish sausage "Chorizo" | − |
| *Debaryomyces fabryi* | PR 18[a] | Meat | − |
| | PR 66[a] | Spanish sausage "Chorizo" | − |
| | Qba | Cheese | − |
| *Debaryomyces hansenii* | CH2[a] | Spanish sausage "Chorizo" | + |
| | CH3[a] | Spanish sausage "Chorizo" | + |
| | CH4[a] | Spanish sausage "Chorizo" | + |
| | CYC 1265 | Canned aubergine | + |
| | EPEC 1.3[b] | Cheese | + |
| | EPEC 2.1[b] | Cheese | + |
| | EPEC 2.2[b] | Cheese | + |
| | EPEC 3.1[b,d] | Cheese | + |
| | EPEC 4[b] | Cheese | + |
| | EPDI 6[b,c] | Cheese | − |
| | Es 4[a] | Marzipan | + |
| | J-1[a] | Iberican ham | + |
| | J-9[a] | Iberican ham | + |
| | J-11[a] | Iberican ham | + |
| | J-12[a] | Iberican ham | + |
| | J-14[a] | Iberican ham | + |
| | J-15[a] | Iberican ham | + |
| | J-16[a] | Iberican ham | + |
| | J-17[a] | Iberican ham | + |
| | J-18[a] | Iberican ham | + |
| | J-19[a] | Iberican ham | + |
| | J-20[a] | Iberican ham | + |
| | 3C1.1[b] | Cheese | + |
| | 3L1.1[b] | Cheese | + |
| | 3I1.1[b] | Cheese | + |
| | 3hgE[b] | Cheese | + |
| | 29C1.1[b] | Cheese | + |
| | 29C1.2[b] | Cheese | + |
| | 29C2p[b] | Cheese | + |
| | 29I1.2[b,d] | Cheese | + |
| | 29 Inf 1[b] | Cheese | + |
| | PR5[a] | Spanish sausage "Chorizo" | + |
| | PR11[a] | Spanish sausage "Chorizo" | + |
| | PR 13[a] | Meat | + |
| | Yaa[b,c] | Spanish pastry "Pionono" | − |
| *Issatchenkia orientalis*[e] | PR 3[a] | Cheese | − |
| *Meyerozyma guilliermondii* | Mi1[a,b] | Jam | - |
| *Torulaspora delbrueckii* | CYC 1176[a,b] | Plain yogurt | − |
| *Wickerhamomyces anomalus*[f] | MA 11.2[a,b] | Jam | − |
| | TYN 1.3[a,b] | Nougat | − |
| *Zygosaccharomyces rouxii* | T2R[a,b] | Nougat | − |

[a] Identified by PCR-RFLP analysis of the IGS region of the rDNA.
[b] Identified by RFLPs of the 5.8S-ITS rDNA region.
[c] The D1/D2 26S rDNA gene sequences of these strains showed 100% sequence similarity with *Debaryomyces fabryi* CBS 789[T].
[d] The D1/D2 26S rDNA gene sequences of these strains showed 100% sequence similarity with *Debaryomyces hansenii* NRRLY-7426.
[e] Now *Pichia kudriavzevii* (Kurtzman et al., 2011).
[f] Formerly *Pichia anomala* (Kurtzman and Fell, 1998).

## 2. Materials and methods

### 2.1. Strain and culture conditions

A total of 94 strains were used in this work. Tables 1 and 2 show the yeast strains obtained from different Type Culture Collections, strains previously isolated and identified in our laboratory (Quirós et al., 2005; Romero et al., 2005) and strains recently identified for this work by PCR-RFLPs of the 5.8S-ITS rDNA region (Esteve-Zarzoso et al., 1999) using primers ITS1 and ITS4 (White et al., 1990) and restriction enzymes *Hinf*I, *Hae*III and *Cfo*I. The patterns obtained were compared with the Yeast-id database (http://www.yeast-id.com). All strains were cultured at 28 °C in Yeast Morphology Broth and routinely maintained in the same culture medium plus Agar (YMA): 0.5% (w/v) yeast extract (Difco Laboratories, Detroit, Michigan, USA), 0.3% (w/v) Proteose-Peptone No. 3 (Difco), 0.3% (w/v) malt extract (Difco), 1% (w/v) glucose (Panreac Quimica S.A., Barcelona, Spain), and 2% (w/v) agar.

### 2.2. Primer design

The putative *PAD1* homologous region (729 bp) present in *D. hansenii* was used for primer design. The sequences were obtained from NCBI. The forward primer DhPadF, 5′ GCGACTATGAACAGGTTTCC AACGA 3′, was selected from nucleotides 101 to 125 and the reverse primer DhPadR, 5′CCTTCAATGTAACATCAGCGGCCC 3′, from nucleotides 479 to 502 bp. Hairpin formation, 3′complementarity and potential self-annealing sites were tested by Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). The primers used were prepared by Conda Labs—Spain.

#### 2.2.1. DNA template preparation and PCR conditions

The DNA template was obtained following the protocol described by Lõoke et al. (2011) or from fresh colonies. PCR amplifications were performed in three different thermocycles and each strain was tried at least twice. The DNA amplifications were carried out in 25 μL reaction volume containing 100 ng of template DNA, 1.25 μL of each primer (20 μM), 2.5 μL of 10 × PCR buffer, 1 μL of MgCl2 (50 mM), 0.2 μL of dNTPs (100 mM) and 0.2 μL of Taq DNA polymerase (5 U/μL) supplied by the manufacturer (Biotools, Madrid, Spain). Different annealing temperatures were tested, the lowest being 52 °C and the highest 68 °C. PCR conditions were as follows: initial denaturalization at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 45 s at the $T_m$ selected, 72 °C for 45 s; and then 1 cycle of 72 °C for 8 min. PCR-amplified DNA fragments were separated in 1% (w/v) agarose gels (Bio-Rad) stained with 0.05% (v/v) ethidium bromide (Bio-Rad) and visualized under UV light. The GeneRuler 100 bp Plus DNA Ladder (MBI Fermentas) was used as a molecular size marker.

## 3. Results

The primer pair designed for *D. hansenii* was based on the *S. cerevisiae PAD1/YDR538W* gene sequence (http://www.yeastgenome.org). Clustal Omega (McWilliam et al., 2013) was used for primer alignment. The target sites of the primers are sequences flanking a putative homologous region of the *PAD1* gene. This region presents a homology of 69% with the *PAD1* gene in *S. cerevisiae*. Subsequently, the suitability of the primers was confirmed by PCR amplification on the yeast strains listed in Table 1. We have tested the DhPadF/DhPadR primers in three different thermocycles and the results did not depend on the PCR machine. It is important to note that at every annealing temperature assayed, a positive result was obtained only with *D. hansenii*. By contrast, the closest outgroup, *D. fabryi*, gave negative results. The best result was obtained after 30 cycles and with an annealing temperature of 67 °C. A clear single fragment about 400 bp was produced by all *D. hansenii* strains. The remaining species of *Debaryomyces*, as well as other yeast species commonly found in foodstuffs, were not amplified with the *D. hansenii* specific primers (Table 1, Fig. 1). In addition, the specificity of the PCR assay developed in this work was tested on i) yeast strains previously identified in our laboratory by RFLP of the IGS region (Quirós et al., 2006; Romero et al., 2005), and ii) on other isolates from several contaminated or spoiled foods identified by PCR-RFLP-5.8S ITS in this work, marked in Table 2 with an [a]. All the *D. hansenii* strains gave positive results with amplicons of 400 bp (Table 2) with two exceptions; *D. hansenii* EPDI6 and *D. hansenii* Yaa, identified by PCR-RFLP-5.8S ITS. To confirm the identification the D1/D2 domain of 26SrDNA has been

sequenced. The results confirmed that these two strains do not belong to *D. hansenii* and should instead be included in the species *D. fabryi*. Neither *D. fabryi* nor *D. subglobosus* profiles are included in the yeast-id data base which complicates their identification. Fig. 2 shows the identical profiles obtained by *Hae*III PCR-RFLP-5.8S ITS for *D. hansenii*, *D. fabryi* and *D. subglobosus* for the strains EPDI6 and Yaa. Identical profiles for all strains were also obtained with *Cfo*I and *Hinf*I (data not shown).

## 4. Discussion

In some laboratories, common techniques such as the RFLPs of the 5.8S ITS rDNA region are a routine practice (Padilla et al., 2014). In this work we confirm that these RFLPs produce the same profiles in *D. hansenii*, *D. fabryi* and *D. subglobosus* and therefore lead to misidentifications.

Previously, in our laboratory we have been able to identify *D. hansenii* and to discriminate among *Debaryomyces* species (Quirós et al., 2006; Romero et al., 2005) through PCR-RFLP of the IGS region (rDNA), which, although precise, is more expensive and time consuming than a rapid identification with species-specific primers.

The putative *PAD1* homologous sequence of *D. hansenii* species, especially in the primer regions, is quite different across available yeast species. Namely, when the sequence on which the primers were designed was introduced in the nucleotide-BLAST it only detected itself on the genomic copy, LOCUSXM_46154. Unfortunately, close outgroups are not available.

The primers DhPADF/ DhPADR developed in this assay produced a clear single fragment of 400 bp in all of the *D. hansenii* strains tested; no false negatives were detected. These primers always identify *D. hansenii* irrespective of the temperature used. Nevertheless, the optimal temperature for this new assay is 67 °C (a restrictive temperature) because at this temperature we obtained more defined bands. On the other hand, no false positives were found in the other 22 species from Culture Collections such as *Torulaspora*, *Hanseniaspora*, *Zygosaccharomyces* that are commonly found in foods or from other origins (Tables 1 and 2 and Fig. 1). In fact, it was remarkable to verify that no amplification was obtained in *D. fabryi* (formerly *D. hansenii* var *fabryi*), *D. subglobosus* (formerly *D. hansenii* var *fabryi*) as well as in other yeast species formerly included in the genus *Debaryomyces* (Kurtzman and Fell, 1998). The revision of the genus addressed recently (Kurtzman et al., 2011) makes it necessary to review the identity of many strains in private collections. Common methods may lead to mistakes and for modest laboratories sequencing a large number of strains can be expensive and slow. Also, it is important when the search is directed at one species, in this case *D. hansenii*, which may be a small fraction of all isolated yeasts.

As mentioned in the Introduction, RFLPs of the 5.8S-ITS rDNA region provided excellent results for other species, but did not differentiate between *D. hansenii* and *D. fabryi*, as we confirmed in work previously reported by Cano-Garcia et al. (2013) for *D. fabryi* and for other *Debaryomyces* species (Martorell et al., 2005). In fact, two misidentifications have been detected with this new method; EPDI6 and Yaa have now been confirmed by sequencing as *D. fabryi* strains. Little is known

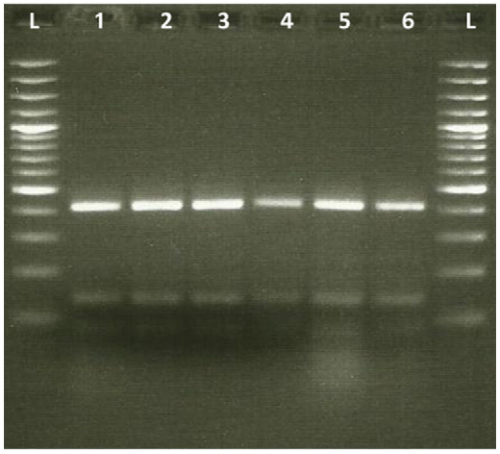


**Fig. 2.** ITS-PCR RFLP profiles of *Debaryomyces* strains digested with restriction enzyme *Hae*III. L: 100 bp ladder. Lane 1: *D. fabryi* EPDI.6, Lane 2: *D. hansenii* EPEC1.3, Lane 3: *D. fabryi* Yaa, Lane: 4 *D. subglobosus* CBS 792$^T$, Lane 5: *D. hansenii* CECT11369$^T$, and Lane 6: *D. fabryi* CECT11370$^T$.

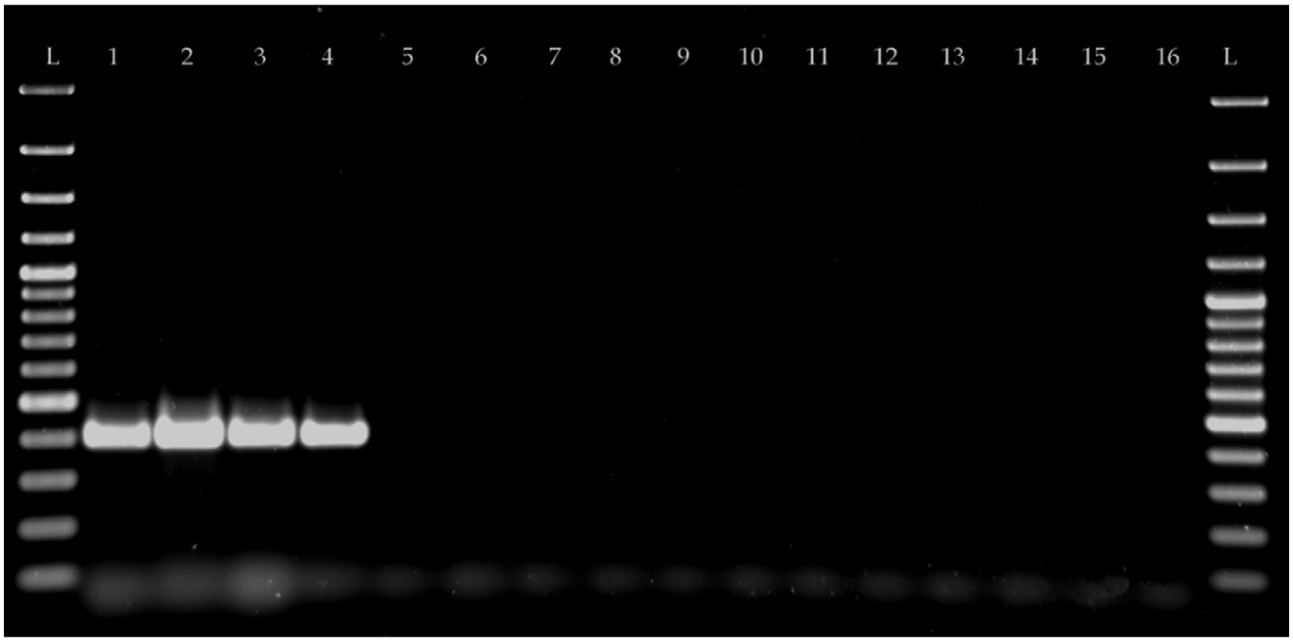


**Fig. 1.** Amplification results obtained with primer pair DhPADF/DhPADR. L: 100 bp ladder. Lanes 1–4: *Debaryomyces hansenii* CECT 10414, CBS 1792, CBS 164, CBS 1102, respectively. Lanes 5–16: *D. fabryi* CECT 11365, *D. maramus* CBS 4264, *Zygosaccharomyces bailii* CECT 1898$^T$, *Z. fermentati* CECT 11056$^T$, *Meyerozyma guilliermondi* Mi1, *Yarrowia lipolytica* CECT 10363, *Candida cretensis* CECT 12038, *Hanseniaspora uvarum* CECT 11105, *Torulaspora delbrueckii* CBS 1146$^T$, *Kluyveromyces lactis* CECT 10361, *Issatchenkia orientalis* PR3 and negative control, respectively.

about the ecology of *D. fabryi*, the majority of the isolates come from clinical sources, but around 40% come from foods (Kurtzman et al., 2011; Wrent et al., 2014). Our strains (EPDI6 and Yaa) contribute to increasing this number.

Furthermore, the primers also recognized *D. hansenii* CBS 766, the identity of which was questioned by Gente et al. (2007) because the primer design by them failed to amplify in this strain.

The primers developed in this work can be used directly on colonies with 100% success rate. This would save users considerable time. However, we recommend the DNA extraction method described by Lõoke et al. (2011) because the extracted DNA can also be stored and used for future PCR amplifications. No enzymes are required or extreme temperatures, and in 15 min the DNA extracted is suitable for PCR amplification of a large variety of yeasts.

Routine identification should be fast and reasonably priced. The assay here proposed is a rapid and affordable method that enables the identification of *D. hansenii* among all of the strains isolated from different foodstuffs and, in addition, institutional or individual culture collections might use this assay to achieve a rapid confirmation or re-identification of *D. hansenii* strains.

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# Capítulo 5

**Development of an affordable typing method for *Meyerozyma guilliermondii* using microsatellite markers.**

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*Development of an affordable typing method for Meyerozyma guilliermondii using microsatellite markers.*

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Keywords:

Microsatellite typing; *Meyerozyma (Pichia) guilliermondii*; *M. caribbica*; Spoilage; Foods; Wine

Highlights:

- Microsatellite markers have been analyzed for the first time in *Meyerozyma guilliermondii.*
- A single typing method has been developed for intra-specific discrimination in *Meyerozyma guilliermondii* species.
- High reproducibility and specificity of the microsatellite tool for *M. guilliermondii*.
- Three microsatellite markers were sufficient to produce an intra-specific differentiation.
- One microsatellite-marker amplified *M. caribbica*.

Abstract

Despite previously published methods, there is still a lack of rapid and affordable methods for genotyping the *Meyerozyma guilliermondii* yeast species. The development of microsatellite markers is a useful genotyping method in several yeast species. Using the Tandem Repeat Finder Software, a total of 19 microsatellite motifs (di-, tri- and tetra- repetition) were found *in silico* in seven of the nine scaffolds published so far. Primer pairs were designed for all of them, although only four were used in this work. All microsatellite amplifications showed size polymorphism, and the results were identical when repeated. The combination of three microsatellite markers (sc15F/R, sc32F/R and sc72F/R) produced a different pattern for each of the Type Culture Collection strains *of M. guilliermondii* used to optimize the method. The three primer pairs can be used in the same PCR reaction, which reduces costs, in tandem with the fluorescent labeling of only the forward primer in each primer pair. Microsatellite typing was applied on 40 more *M. guilliermondii* strains. The results showed that no pattern is repeated between the different environmental niches. Four *M. guilliermondii* strains were only amplified with primer pair sc32 F/R, and subsequently identified as *Meyerozyma caribbica* by *Taq* I-RFLP of the 5.8S ITS rDNA. Most out-group species gave negative results even for physiologically similarly species such as *Debaryomyces hansenii*. The microsatellite markers used in this work were stable over time, which enables their use as a traceability tool.

## 1 Introduction

The *Pichia* species forming coenzyme Q-9 were recently assigned to new clades, five of which were proposed as new genera. One of these is *Meyerozyma*, which includes the old species *Pichia caribbica* and *Pichia guilliermondii,* now named *M. caribbica* and *M. guilliermondii* (Kurtzman and Suzuki, 2010). *M. guilliermondii* is a ubiquitous species with strains isolated worldwide (Kurtzman, 2011*).* Due to its widespread distribution, it is found as a contaminant in a variety of foods like soft drinks, fruit juice, dairy products, and some fermented products such as wine, beer, and cocoa, where it can cause spoilage and consequently important economic losses (Deák, 2008; Fröhlich-Wyder, 2003; Schwan and Wheals, 2003). This yeast species is of particular biotechnological interest due to the overproduction of vitamin B2 (riboflavin) and the bioconversion of xylose in the sweetener xylitol (Papon *et al*., 2013; Zou *et al*., 2010), and for the biological control of post-harvest fungal spoilage (Droby *et al.*, 1997; Lahlali *et al.*, 2011; Lima *et al.*, 2013; Petersson and Schnurer, 1995; Wszelaki and Mitcham, 2003; Zhao *et al.*, 2008). It has recently been proposed as a starter in dough fermentation, thereby extending shelf-life by delaying fungal growth on baked goods (Coda *et al*., 2013).

Given its wide area of interest – and notwithstanding several previously published methods – there is still a lack of reliable and accurate approaches for genotyping *M. guilliermondii*. Few methods have been described for strain discrimination in *M. guilliermondii.* Martorell *et al*. (2006) used mtDNA restriction analysis to characterize strains based on their different efficiencies of 4-ethylphenol production (off-flavor in wine). Romi *et al*. (2014) reported that this method differentiated between *M.*

*guilliermondii* and *M. caribbica* but failed to differentiate strain levels. Similarly, the use of RAPD in combination with killer toxin biotypes succeeded in detecting potential spoilage strains (Lopes *et al*., 2009), where *M. guilliermondii* produced high levels of 4-vinylphenol and 4-vinylguaiacol in Patagonian wines. However, some authors consider that RAPD may cause artefactual amplification (Albertin *et al*., 2014). Recently, Corte *et al*. (2015) used a different approach to distinguish clinical strains of *M. guilliermondii* ( anamorph, *C. guilliermondii*). They analyzed several methods (i-SSR $(GACA)_4$, RAPDs, FTIR) and succeeded in differentiating isolates of the fruit and non-fruit group, although none of the tools were able to discriminate between clinical and environmental isolates within the non-fruit group.

The microsatellite markers is a useful genotyping method at strain level in *Brettanomyces bruxellensis, Candida albicans, Saccharomyces cerevisiae,* and *Saccharomyces uvarum*, as it is fast, easy, and highly polymorphic (Albertin *et al*., 2014; Antonangelo *et al*., 2013; Hennequin *et al*., 2001; L'Ollivier *et al*., 2012; Zhang *et al*., 2015). Microsatellites were first genotyped by Litt and Luty (1989). They are tandem repeat motifs consisting of one to six nucleotides present in the genomes of all eukaryotes.

The aim of this work is to develop microsatellite markers as a single typing method for intra-specific discrimination of *Meyerozyma guilliermondii*.

## 2 Materials and Methods

*2.1 Yeast strains and culture conditions*

Seventy-four yeast strains were used in this study (Table 1). Eight *M. guilliermondii* strains were obtained from the CECT (SpanishType Culture Collection); 48 of the remaining yeast strains used for validation belonged to the *M. guilliermondii* species, and 26 to other yeast species, most of which were isolated from foods, dairy products and wine (Table 1). Strains were routinely grown at 28 ºC in YMB and maintained on YMA slants at 4 ºC.

*2.2 DNA extraction*

The DNA templates were obtained by weakening the cell wall by using lithium acetate–SDS (2%) solution and subsequent precipitation of DNA with ethanol as described by Lõoke *et al*. (2011).

*2.3 Identification of microsatellite motifs and primer design*

The Tandem Repeats Finder (TRF) software (Benson, 1999) was used to search for di-, tri- and tetra- nucleotide repeats present in the yeast strain *M. guilliermondii* CBS 566, whose genome sequences were downloaded as scaffolds from NCBI (http//www.ncbi.nlm.nih.gov) (Sayers *et al*., 2009). Nineteen microsatellite markers were designed for target amplifications (Table 2), four of which were assayed for use in this study. The primers were designed using the design program (www.bioinformatics.nl/primer3plus*)*. Each specific forward primer was 5´- tailed with the M13 universal sequence (5´ TT TTC CCA GTC ACG AC 3´) and the universal M13 primer was labelled with the fluorescent dye FAM-6 (Schuelke, 2000). They were prepared by Conda Labs, Spain.

**Table 1**

Yeast species, strains and their respective genotype determined by microsatellites amplification obtained for each *M. guilliermondii* strain.

Numbers indicate allele sizes (bp) for each primer pair

| Species | Collection | Origin | sc15F/R | sc22F/R | sc32F/R | sc72F/R | Genotype |
|---|---|---|---|---|---|---|---|
| *Meyerozyma guilliermondii* | CECT 1456$^{T}$ * | Insect frass on *Ulmus americana* , United States | 371 | 215 | 209 | 250 | A |
| | CECT 1019 | Flower of *Gentiana imbricata* | 355 | 223 | 209 | 244 | B |
| | CECT 1021 | Soil from drilling care , Japan | 371 | 215 | 250 | 302 | C |
| | CECT 1438* | Pozol, Mexican fermented maize dough, Mexico | 388 | 215 | 206 | 250 | D |
| | CECT 10100** | Fruit of *Zizyphus vulgari,* Spain | ( - ) | ( - ) | 224 | ( - ) | |
| | CECT 10157 | Fruit in syrup, Spain | 371 | 215 | 209 | >600 | E |
| | CECT 12791 | Soil, Spain | 388 | 215 | 250 | 262 | F |
| | CECT 12839 | Beer var. Garrafal, Spain | 371 | 215 | 206 | 361 | G |
| *Meyerozyma guilliermondii**** | CYC 1357 | Starch concentration | 355 | | 221 | 247 | H |
| | ISA 2105** | Grapes winery 5, Portugal | ( - ) | | 195/225 | ( - ) | |
| | ISA 2110 | Red wine, winery 4, Portugal | 371 | | 220 | 330 | I |
| | ISA 2125 | Pump outlet, winery, Portugal | 371 | | 209 | 408 | J |
| | ISA 2274 | Winery, Portugal | 371 | | 211 | 271 | K |
| | ISA 2375** | Winery, Portugal | ( - ) | | 212 | ( - ) | |
| | ISA 2376** | Winery, Portugal | ( - ) | | 245 | ( - ) | |
| | Tur 44 | Nougat, Toledo, Spain | 371 | | 206 | 293 | L |
| | 143 Valencia | Oil pollution, Oviedo, Spain | 371 | | 251 | 281 | M |
| | 134 Valencia | Oil pollution, Oviedo, Spain | 371 | | 251 | 284 | N |
| | LO 562 | Grape must (Merlot) 2005, France | 371 | | 220 | 256 | O |
| | LO 652 | Grape must (Merlot) 2006, France | 371 | | 206 | 375 | P |
| | LO 654 | Grape must (Merlot) 2006, France | 371 | | 206 | 375 | P |
| | LO 679 | Grape must (Merlot) 2006, France | 371 | | 209 | 259 | Q |
| | LO 708 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 710 | Grape must (Merlot) 2007, France | 388 | | 206 | 235 | R |
| | LO 721 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 728 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 758 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 779 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 783 | Grape must (Merlot) 2007, France | 388 | | 206 | 235 | R |
| | LO 786 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | LO 7140 | Grape must (Merlot) 2007, France | 371 | | 209 | 259 | Q |
| | Mi2 | Strawberry jam, 2007, Spain | 371 | | 209 | 262 | S |
| | Mi3 | Strawberry jam, 2007, Spain | 371 | | 209 | 262 | S |
| | Mi4 | Strawberry yogurts, 2007, Spain | 371 | | 209 | 268 | T |
| | Mi5 | Strawberry yogurts, 2007, Spain | 371 | | 209 | 268 | T |
| | Mi6 | Strawberry yogurts, 2007, Spain | 371 | | 209 | 268 | T |
| | Mi7 | Berry yogurts, 2007, Spain | 371 | | 209 | 268 | T |
| | Mi8 | Berry yogurts, 2007, Spain | 371 | | 209 | 268 | T |
| | YFB1 | Plain yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YN2 | Plain yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YFB4 | Plain yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YN5 | Strawberry yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YF6 | Strawberry yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YFB7 | Berry yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YN8 | Berry yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | YF9 | Berry yogurts, 2008, Spain | 371 | | 209 | 268 | T |
| | Ya10 | Apricot yogurts, 2008, Spain | 371 | | 209 | 262 | S |
| | Ma11.1 | Apricot jam, 2008, Spain | 371 | | 209 | 262 | S |
| **Other Yeast species** | | | | | | | |
| *Candida carpophila* | MUCL 029964 | Insect | ( - ) | 215 | 200/600 | ( - ) | |
| *Candida cretensis* | CECT 12038 | Pork sausage | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces coudertii* | CBS 5167 $^{T}$ | Droppings of *Aptenodytes patagonica* | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces fabryi* | CBS 1796 | Erythematous-squamatic skin lesion | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces fabryi* | CECT 11370$^{T}$ | Interdigital mycotic lesion | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces hansenii* | CECT 11369$^{T}$ | Carlsberg Laboratories | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces maramus* | CECT 11362$^{T}$ | Atmosphere | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces melissophilus* | CECT 11410$^{T}$ | Gut of *Apis mellifica* var. *adonsonii* (honey bee) | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces prosopidis* | CBS 8450$^{T}$ | Exudate of *Prosopis juliflora* (mesquite tree) | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Debaryomyces udenii* | CBS 7057 | Soil | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Hanseniaspora uvarum* | CECT 10142 | Cucumber | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Klyveromyces lactis* | CECT 10361 | Cheese | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Kregervanrija delftensis* [a] | CECT 10238$^{T}$ | Cider | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Lachancea cidri* [b] | CECT 10657 $^{T}$ | Cider | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Lachancea fermentati* [c] | CECT 11056$^{T}$ | Sediment of pepermint | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Pichia fermentans* | CECT 1455$^{T}$ | Buttermilk | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Pichia membranifaciens* | CECT 1115$^{T}$ | Elm exudate | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Saccharomyces cerevisiae* | ATCC 7754 | Fleischmann bakers yeast | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Schwanniomyces pseudopolymorphus* [c] | CECT 11360$^{T}$ | Tanning fluid, prepared from bark of sweet-chestnut | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Schwanniomyces yamadae* [e] | CBS 7035$^{T}$ | Soil, of grassland | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Torulaspora delbrueckii* | ATCC 66821$^{T}$ | Unknown | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Wickerhamomyces anomalus* [f] | CECT 12806 | Grape juice | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Yarrowia lipolytica* | CECT 1240 | Maize-processing plant | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Zygosaccharomyces bailii* | CECT 1898$^{T}$ | Unknown | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Zygosaccharomyces mellis* | CBS 1091 | Fermenting honey | ( - ) | ( - ) | ( - ) | ( - ) | |
| *Zygosaccharomyces rouxii* | CECT 10633 | Honey | ( - ) | ( - ) | ( - ) | ( - ) | |

ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures; CECT: Colección Española de Cultivos Tipo;
ISA: Instituto Superior de Agronomía, Lisboa, Portugal; MUCL: Belgian Coordinated Collections of Microorganism.
[T]: Type strain
[*]Assayed strains maintained in two different laboratories
** Strains identified in this work as *M.caribbica* following Romi *et al*. (2014)
*** Strains used for validation
[a]Formerly *Pichia delftensis* (Kurtzman, 2006)
[b]Formerly *Zygosaccharomyces cidri (* Kurtzman, Fell and Boekhout, 2011)
[c]Formerly *Zygosaccharomyces fermentati* (Kurtzman, Fell and Boekhout, 2011)
[d]Formerly *Debaryomyces pseudopolymorphus* (Kurtzman and Fell, 1998)
[e]Formerly *Debaryomyces yamadae* (Kurtzman and Fell, 1998)
[f]Formerly *Pichia anomala* (Kurtzman and Fell, 1998)

### *2.4 Microsatellites amplifications*

The DNA amplifications were done with 50-100ng of genomic DNA, 1.25 µL of reverse primer and M13-labelled primer (10 µM), 0.125µL of M13-forward primer (10µM), 12.5µL NZYtaq2x colourless Mastermix, and nuclease-free water to a final volume of 25µL. The touchdown PCR amplification (Korbie and Mattick, 2008) protocol used for *M. guilliermondii* was as follows: 1 cycle of 1 min at 94ºC, 10 cycles of 30s at 94ºC, 30s at 60ºC (after each cycle the annealing temperature was decreased by 1ºC) and 30s at 72ºC; then 20 cycles of 30s at 94ºC, 30s at 50ºC and 30s at 72ºC; and the final extension step of 2 min at 72ºC. The amplifications were performed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Each strain was tested at least twice.

The PCR products were separated on a 1.5% agarose gel, Biorad, USA (Fig. 1). The PCR products with different sizes can be mixed and measured. The microsatellite PCR product was measured on an ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) using the GeneScan™ 500 LIZ® Size Standard marker 30-600bp (Life technologies). At least two repetitions were performed for each strain.

*2.5 ITS-RFLP*

Following the method previously described by Romi *et al*. (2014) for the differentiation of *M. guilliermondii* and *M. caribbica*, the ITS1-5.8S-ITS2 region was amplified in all strains using primers ITS1 ( 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') (White *et al*., 1990). The PCR product (5 µL) was digested with 0.5 µL of *Taq* I endonuclease (Life technologies) in a 10 µL reaction volume at 65 °C for 16 h. Restriction fragments were separated by electrophoresis on 2.5% (w/v) agarose gels stained with 0.05% (v/v) ethidium bromide. The 100bp DNA ladder (MBI Fermentas) was used as a molecular size marker.

*2.6 DNA sequencing and sequence analysis*

To clarify the identity of four strains CECT 10100, ISA 2105, ISA 2375 ISA2376, the D1/D2 domains of the 26S rDNA were amplified using primers NL1 (5'-GCATAT CAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman & Robnett, 1998). PCR products were cleaned using the Ultraclean™ PCR clean-up kit (MO-BIO, Larsband, USA). PCR products were sequenced by STAB Vida Lda. (Portugal) using an ABI 3730XL sequencer (Applied Biosystems, USA). Sequences were edited with the EditSeq program included in DNAstar 7.1 software (Lasergene, USA). Each set of sequences was aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). The edited sequences were compared with those previously available on the NCBI databases using the BLAST program.

**Table 2** Motifs identified by Software Tandem Repeat Finder in *Meyerozyma guilliermondii* and primers designed

| Scaffold | Microsatellite name | Motifs | Copy number | Mismatches | Indices with flanking sequences | Primer F | Primer R | Product size (bp) |
|---|---|---|---|---|---|---|---|---|
| 1 | sc11 | TGT | 16 | 6 | 287,206--287,253 | TGTAACTGACCAGGGTGCTG | ACCAGTGCAGGAAGCCATAC | 298 |
| | sc12 | GAT | 9.7 | 0 | 1,033,042--1,034,070 | ATCGGAAGAGGTCGGAAAGT | GCGGACCAAGGTGTACTTGT | 265 |
| | sc13 | GAC | 18.7 | 8 | 1,051,135--1,052,190 | GAAGAAGAAACTGCCGATGC | AACCCACTAGCTCCCCAACT | 222 |
| | sc14 | GAA | 23.7 | 11 | 1,252,737--1,253,807 | CCTCTTGAACTTCGGACTCG | ATGAAGAAGCTGCCGAAGAA | 243 |
| | sc15 | **AG** | 44.5 | 12 | 1,670,917--1,672,000 | **AGGAAATGGTGGACGACAAG** | **TGAGTAGGGCTTGCACACTG** | 359 |
| 2 | sc21 | GCT | 12 | 2 | 573,012--574,047 | GTTTTTCAACCGAGCCTGAG | CATGGCAGGTCAGCAGAGTA | 277 |
| | sc22 | **AAAG** | 21.5 | 8 | 1,027,115--1,028,210 | **CTCTGAGAACGGGACCCATA** | **AGACATCGATGCCAAAGGAT** | 203 |
| 3 | sc31 | AGG | 9.7 | 0 | 796,451--797,479 | GCGCTTGGTAGATTTTGAGC | TCGTCAAACTTCGTCACCAG | 181 |
| | sc32 | **TCA** | 20.7 | 10 | 1,670,633--1,670,694 | **GCGTCCTTATCGTCTTCGTC** | **ATGGGTGGATATCGTGGAAA** | 189 |
| 4 | sc41 | AGA | 14 | 6 | 66,340--67,381 | CCAAGCCGATCCTACACCTA | CAAGCGAAGCTTTCTCTCGT | 220 |
| 5 | sc51 | TTC | 10 | 0 | 400,557--401,086 | AGAAACAGCCTCGGGAATTT | ACCGGTATTGAAGGCAGTTG | 205 |
| | sc52 | GTT | 14.3 | 6 | 709,950--710,992 | TGCTATTCCTCGCCAGACTT | CAGCAACAGAGCCGTATCAA | 215 |
| | sc53 | GAA | 14 | 4 | 864,217--865,258 | TGTGGTCGTTGTCGAATGTT | CGACGCCTTTGAATACAACC | 237 |
| 6 | sc61 | GAA | 15 | 8 | 633,712--634,756 | TGCTTCTGGGTTCTCGAAGT | ATACTGGATCCACCGAGTGC | 275 |
| | sc62 | AGA | 20.7 | 5 | 734,850--735,908 | GCAAGCCGCAACTTGTAGA | CTCACTGTTGGTGCAGGAGA | 270 |
| | sc63 | CAA | 9.3 | 0 | 942,241 --943,268 | GTAGGATTCCCCACCTCCAT | CTTTGGGTCGAGGGTAGCTT | 240 |
| 7 | sc71 | GAA | 12.3 | 2 | 322,801--323,837 | CAAAAGAGATGCGTGAAACG | TCCTGTTCCTCTGTCGGTTT | 150 |
| | sc72 | **CTT** | 40 | 6 | 641,617--642,737 | **ACCATAGAATGAGCGGTAGCA** | **TTTCTGTTCCAAGGCCAAAG** | 259 |
| | sc73 | AAG | 23 | 11 | 649,844--650,913 | CTGAGATGTTGGACGAAGCA | TGTATTTGTCGGTGGCTTGA | 273 |
| 8 | | None detected | | | | | | |
| 9 | | None detected | | | | | | |

Primes and motifs used in this work are in **bold**

## 3 Results

*3.1 In silico selection of microsatellite markers for M. guilliermondii*

Using the TRF software, a total of 19 microsatellite motifs were found *in silico* within the sequences published so far (9 scaffolds) for *M. guilliermondii* (Table 2). Microsatellites were located on 7 different scaffolds, with a copy number that ranged from 9.3 to 44.5. Most loci were in non-coding regions, except those that encode for a putative flavoprotein sc13 (XP_001487219), Gtr1/RagA G sc63 (protein conserved region (XM_001483126), and nuclear pore complex subunit Nro1 sc73 (XM_001482466).

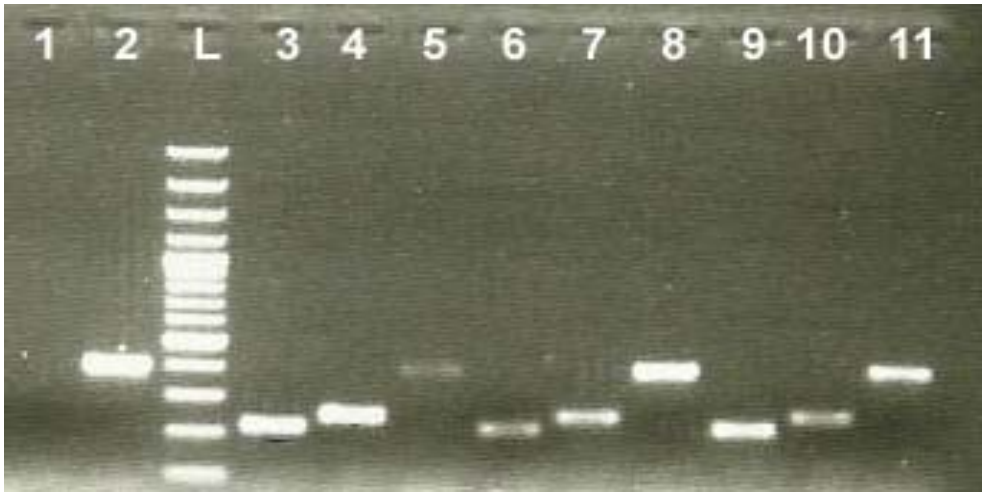


**Fig. 1** Microsatellite amplification with primer pairs sc15F/R, sc22F/R and sc32F/R in the yeast strain CECT 12791, repeated thrice. 1: Negative control, 2: sc15 F/R, L: 100bp ladder, 3: sc22F/R, 4: sc32F/R, 5: sc15F/R, 6: sc22F/R, 7: sc32F/R, 8: sc15F/R, 9: sc22F/R, 10: sc32F/R, 11: sc15F/R

*3.2 Evaluation of selected microsatellite markes on Spanish Type Culture Collection (CECT) strains*

Although primers were designed *in silico* for all the motifs (Table 2), only four –sc15F/r, sc22F/R, sc32F/R and sc72F/R (Table 2, in bold) – were

assayed for PCR optimization on the collection strains of *M. guilliermondii* listed in Table 1. The amplifications were carried out and the amplicons were separated as described in Material and Methods. As shown in Table 1, all the strains from CECT were successfully amplified with all the primers selected except for strain CECT 10100, which failed for three of the four primers assayed. However, when we verified the identification of all the strains used in this study, as previously described by Romi *et al*. (2014) (data not shown), we were able to confirm that the strain CECT 10100 actually belongs to *M. caribbica*, as revealed by the characteristic profile of *Taq*I ITS-RFLP. The sc15, sc22 and sc72 microsatellite motifs may therefore be specific to *M. guilliermondii.* All microsatellites showed size polymorphism, and the results were identical when repeated (Fig.1). Moreover, when assayed two strains CECT 1456$^{T}$ (CBS 2080$^{T}$) and CECT 1438 (CBS6557) (Table 1) conserved for years in our laboratory and another laboratory, the results were consistent, independent of the laboratory and the number of subcultures.

The combination of the three microsatellite markers (sc15R/F, sc32R/F and sc72R/F) produces a different pattern for each of these strains.

*3.3 Microsatellite markers validation*

In view of the discriminatory power obtained for microsatellite markers – and in order to validate the above results – we assayed the combination of these three primers on 66 yeast strains comprising 40 strains of *M. guilliermondii* – mostly related to food, dairy products, or wine – and 26 different strains of other yeast species (Table 1). We observed that i) as expected, with the exception of *C. carpophila,* all the strains belonging to species other than *M. guilliermondii* gave negative results; ii) three *M. guilliermondii* strains from the ISA collection gave negative results; once

again, the non-amplified strains were confirmed as *M. caribbica* by *Taq*I 5.8S ITS RFLP (Fig. 2); iii) there was no common pattern among the strains from different origins, such as strains from wine and grapes (Portugal), a vineyard (France), nougat (Spain), an organic yogurt producer (Spain), or other origins such as oil pollution (Spain), among others.

Within the same ecological niche – the vineyard (France) and the yogurt producer (Spain) – some strains with the same pattern were isolated up to 1 year later. For example: *M. guilliermondii* LO679 isolated in 2006 was isolated again in 2007 (LO 708), etc.

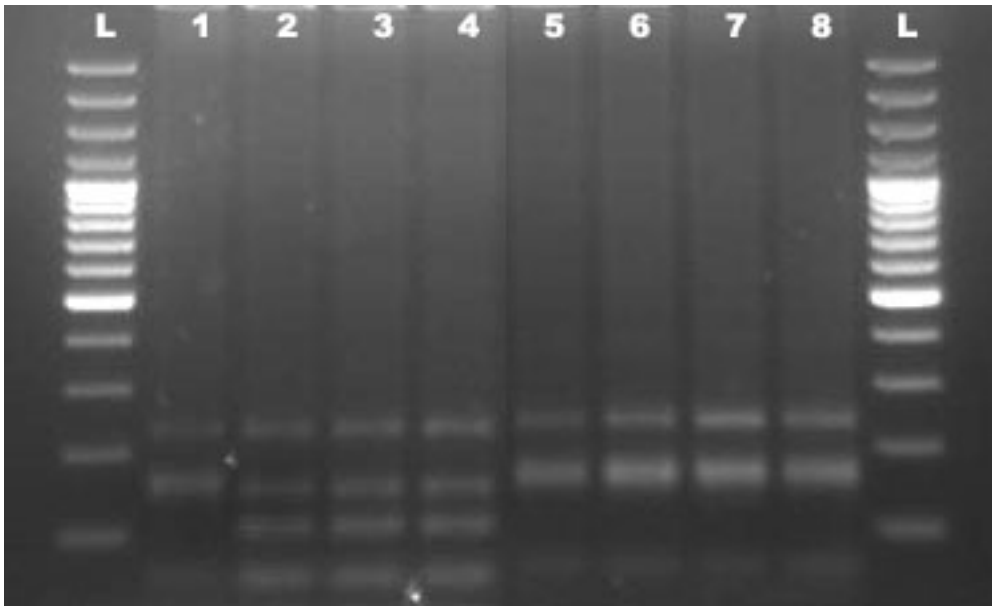


**Fig. 2** ITS-PCR-RFLP profiles of *Meyerozyma guilliermondii* strains digested with restriction enzyme *Taq*I. L: 100bp ladder. Lane 1: *M. guilliermondii YFB1*, Lane 2: *M. guilliermondii* ISA 2376, Lane 3: *M. guilliermondii* ISA 2375, Lane: 4 *M. guilliermondii* ISA 2105, Lane 5: *M. guilliermondii* CECT 2021, Lane 6: *M. guilliermondii* CECT12839, Lane7: *M. guilliermondii* ISA 2110, Lane 8: *M. guilliermondii* CECT 1438.

## 4 Discussion

Strain typing has been used in the industrial context for several different purposes: for discriminating between different biotypes with specific properties, for monitoring the behavior of an industrial strain, and for tracing a single strain to determine the source of contamination – for example, as a means of solving spoilage problems (Wrent *et al*., 2010). However, a correct strain typing method requires a previous and accurate identification. In this work we have developed a method based on microsatellites. Microsatellite genotyping is widespread in population-based, ecological and evolutionary studies of various species (Rosenberg *et al*., 2002; Schlötterer, 2001), for tracing beef products and for certifying the origin and identity of dairy products (Sardina *et al*., 2015; Shackell *et al*., 2005). Microsatellites have the advantage of being highly polymorphic and portable, meaning that genotyping can be compared across different laboratories (Albertin *et al*., 2014). The *M. guilliermondii* genome is not assembled. However, there are nine available scaffolds that allowed us to identify the microsatellite loci. We searched for the most frequent motifs present in yeasts (Lim *et al*., 2004). Two out of the nine scaffolds studied did not present any detectable microsatellite (Table 2). It has previously been reported that microsatellites are less frequent in fungal genomes and – if present – they are normally shorter than those in other taxa (Tóth *et al*., 2000).

The microsatellite markers used in this work were stable through time, as demonstrated by the results mentioned above. The two strains CECT $1456^{T}$ (CBS $2050^{T}$) and CECT 1438 (CBS 6557) did not undergo any detectable mutation in the microsatellite motifs.

Our method permits the combination of the three primers (sc15F/R, sc32F/R and sc72F/R) in the same PCR reaction, which reduces the cost, in tandem with the fluorescent labeling of only one of the primers (Albertin *et al*., 2014; Schuelke, 2000). The method proposed in this work has a very high potential, with 100% discrimination between all the strains assayed from different origins (Table 1). As expected, we found less polymorphism within the same ecological niche, which enabled us to reach some conclusions about the strains (Table 1). For example, the LO 679 strain isolated in a 2006 vintage from a French vineyard is the same strain that was widespread in the vineyard in 2007 (genotype Q); moreover, all jams (M1, M2 and Ma11.1, genotype S) in the yogurt producer are contaminated with the same strain that was present in the apricot yogurt (AY10) 1 year later (Table 1). This demonstrates the consistency of the methods for strains tracing. In this work we have obtained higher discrimination for the yogurt strains than previously with RFLP-mtDNA (Wrent *et al*., 2015). Our method also allowed the discrimination between strains previously assayed by other methods (i- SSR $(GACA)_4$). Namely, the strains CECT 1438 (CBS 6557) and CECT 1021 (CBS 6021) fall in the clade entirely composed of non-fruit strains from various substrates, well discriminated from strains isolated from fruit (Corte *et al*., 2015), but both exhibit the same pattern (Corte *et al*., 2015). However, as can be seen in Table 1, these strains can be differentiated with our method (genotype D and C respectively). There is some uncertainty about the ecology of *M. guilliermondii* due to the unreliability of the phenotypic identification (Kurtzman *et al*., 2011), indicating that many strains identified several years ago could be misidentified and some of the properties attributed to *M. guilliermondii* may need to be reviewed. For example, several strains

used in a work on 4-ethylphenol production (Martorell *et al*., 2006) were also tested in our study. By analyzing all the results together we found that the low-producing strains described by Martorell *et al*. (2006) belong to *M. guilliermondii* (ISA 2110 and ISA 2125), while the high – producing strains (>50 mg/L) included one strain identified in our work as *M. caribbica* (ISA 2105). In the wine industry, levels of 4-ethylphenol over 620 µg/L are considered to produce undesirable aromas (Chatonnet *et al*., 1992), although levels of lower than 400 µg/L have the opposite effect on wine, imparting notes that enhance the aroma (Loureiro and Malfeito-Ferreira, 2003). According to Lopes *et al*. (2009), the *M. guilliermondii* strains (confirmed by sequencing) present in Patagonian wine produced levels of 4-ethylphenol that were too low to be considered dangerous for winemaking. This leads us to suggest that it would be interesting to determine if indeed all high producers belong to *M. caribbica*, and *M. guilliermondii* could therefore be a low- or non-producing 4-ethylphenol species. Some authors in the clinical field (Corte *et al*., 2015; Romi *et al*., 2014) have proposed that further study at strain level is required to differentiate between clinical or food strains, and unravel the pathogenic potential of *M. guilliermondii*. Further research with clinical isolates is needed to study the suitability of this method.

In addition, three of the four microsatellite markers developed were specific for the *M. guilliermondii* species. It should be noted that the method did not amplify species such as *D. hansenii*, a close species (Kurtzman *et al*., 2011) which in some cases has been misidentified as *M. guilliermondii* (Desnos-Ollivier *et al*., 2008). The sc32F/R primer amplifies the phenotypically indistinguishable species *M. caribbica* and *C. carpophila* (Table 1). However, other species belonging to *Meyerozyma* clade such as

*C. smithsonii*, *C athensensis,* and *C. elateridarum* isolated from insects produces a set of unspecific bands that do not interfere with the pattern obtained for *M. guilliermondii* (data not shown). Further studies are required to determine its suitability for the genotyping of *M. caribbica*, whose genome has also been sequenced but is still not assembled. Although it was not among the aims of this work, the combination of selected primers for strain typing succeeded in discriminating both species with a 100% detection rate for *M. guilliermondii*, and allowed us to detect four misidentified strains (CECT10100, ISA 2105, ISA 2375, and ISA 2376), which were confirmed as *M. caribbica* by *Taq*I ITS-RFLP. As according to Romi *et al*. (2014) the D1/D2 sequencing was inconclusive for differentiation of *M. guilliermondii* from *M. caribbica* (data not shown). Although, this permitted us to confirm that these strains belong to *Meyerozyma* genus.

In conclusion, the proposed method is an accurate, reproducible, affordable, fast and unique molecular method for the intra-specific differentiation of *M. guilliermondii.*

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# Capítulo 6

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*Article*

# Assessment of the Factors Contributing to the Growth or Spoilage of *Meyerozyma guilliermondii* in Organic Yogurt: Comparison of Methods for Strain Differentiation

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**Abstract:** In this work we analyze the spoiling potential of *Meyerozyma guilliermondii* in yogurt. The analysis was based on contaminated samples sent to us by an industrial laboratory over two years. All the plain and fruit yogurt packages were heavily contaminated by yeasts, but only the last ones, containing fermentable sugars besides lactose, were spoiled by gas swelling. These strains were unable to grow and ferment lactose (as the type strain); they did grow on lactate plus galactose, fermented glucose and sucrose, and galactose (weakly), but did not compete with lactic acid bacteria for lactose. This enables them to grow in any yogurt, although only those with added jam were spoiled due to the fermentation of the fruit sugars. Fermentation, but not growth, was strongly inhibited at 8 °C. In consequence, in plain yogurt as well as in any yogurt maintained at low temperature, yeast contamination would not be detected by the consumer. The risk could be enhanced because the species has been proposed for biological control of fungal infections in organic agriculture. The combination of the IGS PCR-RFLP (amplification of the intergenic spacer region of rDNA followed by restriction fragment length polymorphism analysis) method and mitochondrial DNA-RFLP makes a good tool to trace and control the contamination by *M. guilliermondii*.

**Keywords:** *Meyerozyma guilliermondii*; *Candida guillermondii*; lactic fermentation; yogurt spoilage; biocontrol

## 1. Introduction

*Meyerozyma Kurtzman et M. Suzuki*, is a new yeast genus that includes the old species *Pichia caribbica* and *Pichia guilliermondii*, now named *Meyerozyma caribbica* and *Meyerozyma guilliermondii*, the type species of the new genus [1]. There is some uncertainty about the ecology of *M. guilliermondii* due to the now-recognized unreliability of the phenotypic identification. From the molecularly confirmed strains, it can be ascertained that it is a ubiquitous species, with strains isolated worldwide from sea water, tree exudates, insects, soil, and foods [2]. It is also included among the 17 ascomycetous yeast species most frequently related to human and animal infections [3]. *M. guilliermondii* in medical microbiology is known as the telemorph of the opportunistic pathogen *Candida guilliermondii*. The biotechnological potential of this species includes its use in organic agriculture for biological control of post-harvest fungal contamination [4–8]. This agricultural application has strong ecological consequences because it implies the inoculation of a significant amount of yeasts cells, specially prepared to survive [9], into the environment. On the other hand, consumer interest in more "natural" foods, not only from organic agriculture but also processed without the addition of chemical preservatives, enhances the probability of microbial growth on this type of foods. Yeasts are not frequent spoiling agents of yogurt because they usually include potassium sorbate, an efficient inhibitor of the yeast microbiota. When sorbate is present the yeast population in yogurt reaches only about $10^4$ CFU/g (Colony Forming Unit) after two months' incubation at 20 °C [10].

In this work, we describe the presence of *M. guilliermondii* in yogurt at high concentrations; we also analyze the factors that favor its presence in this type of environment. In addition, two molecular methods were compared for their suitability for strain discrimination.

## 2. Materials and Methods

### *2.1. Isolation and Culture Conditions*

All strains isolated in this study come from the same industry and are listed in Table 1. In addition, seven reference strains of *M. guilliermondii* from the Spanish Type Culture Collection (CECT) were included for comparison in the typing study (Table 1), as well as a *Saccharomyces cerevisiae* strain as fermentation control. The culture media used were YMB (Yeast morphology broth), YMA (Yeast morphology agar), and YMAC (YMA plus chloramphenicol). YMB had 10.0 g/L glucose (Panreac Química, Barcelona, Spain), 5.0 g/L proteose peptone No. 3 (Difco Laboratories, Detroit, MI, USA), 3.0 g/L yeast extract (Difco), and 3.0 g/L malt extract (Difco). The YMA was YMB solidified with 20.0 g/L agar. YMAC was made by adding 0.5 g/L of Chloramphenicol (Sigma Aldrich Chemie, Steinheim, Germany) to YMA. To isolate the strains, 10 g of the samples were suspended in YMB, homogenized in a Stomacher homogenizer, and serial dilutions in saline solution were made. To enumerate the viable cells, two replicas with four drops (50 μL) of an appropriate dilution were

inoculated on YMAC, following the modified method of Miles and Misra [11,12]. Strains were routinely grown at 28 °C in YMB and maintained on YMA slants at 4 °C.

*2.2. Identification*

The 20 yeast strains isolated in this work were all identified by 5.8S-ITS restriction analysis. The region was amplified using ITS1 and ITS4 primers [13]. For this purpose, the cells were collected from a fresh colony and homogenized in the PCR mixture. The amplified DNA (10 µL) was digested with three restriction endonucleases, *Hinf*I, *Hha*I, and *Hae*III (Amersham Pharmacia Biotech, Buckinghamshire, UK) [14]. The length and number of the fragments obtained with each endonuclease were compared with the yeast ID database (https://www.yeast-id.org) belonging to Spanish Type Culture Collection (CECT). All strains identified as *M. guilliermondii* were subsequently re-identified with the *Taq*I-5.8S-ITS method [15], which allows us to distinguish between *M. caribbica* and *M. guilliermondii*. Subsequently, identification of two selected strains was confirmed by sequencing the 5.8S ITS rDNA region using primers described by White *et al*. [16]

*2.3. Physiological Analysis*

Physiological analysis of growth and fermentation of different carbon sources were carried out as described by Barnett *et al*. and Kurtzman *et al.* [17,18]. Table 2 shows some of them.

*2.4. Methods for Strain Differentiation (Typing)*

Genomic DNA was isolated using the protocol described by Querol *et al*. [19]. For the PCR-RFLP analysis of the Intergenic Spacer region (IGS) of the rDNA, this region was amplified using CNL12 and CNS1 primers (SigmaGenosys, Cambridge, UK) [20] under the conditions described elsewhere [21,22]. Aliquots of PCR amplification products (10 µL) were digested without further purification with endonucleases *Hha*I and *Hap*II (Amersham Pharmacia Biotech, Buckinghamshire, UK). For mtDNA analyses, samples were digested using the restriction endonuclease *Hinf* I (Amersham Pharmacia Biotech, Buckinghamshire, UK), as previously described by Querol *et al.* [19] and modified by López *et al.* [23]. At least two independent analyses were made for each strain (up to five in some of the strains used as controls).

*2.5. Sugar Fermentation*

The fermentation capacities were analyzed quantitatively by ethanol determination. One isolated strain (Mi4) was inoculated in culture media (3.0 g/L of yeast extract (Difco Laboratories, Detroit, MI, USA) and 5.0 g/L of proteose peptone No. 3 (Difco Laboratories, Detroit, MI, USA) with different carbon sources: galactose (1%) or lactate (1%) plus galactose (1%) or sucrose (1%) (Sigma Aldrich Chemie, Steinheim, Germany). After seven days the ethanol produced was measured with Enzytec fluid Ethanol purchased from R-Biopharm, Darmstadt, Germany (Cat. No. E5340), following the instructions supplied by the manufacturer.

**Table 1.** Strains isolated from different samples and strain collection, origin, type of spoilage, and patterns obtained by RFLP mtDNA (Restriction Analysis of the mitochondrial DNA) and by PCR IGS-RFLP.

| Isolated Strains | Identification [a] | Origin | Spoilage | CFU/g | Standard Deviation (Std. dev.) | RFLPs mtDNA | RFELP IGS | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *Hinf*I | *Hha* I (B) | *Hae* III (H) |
| Mi1 | *M. guilliermondii* | Strawberry jam | Bubbles | ND | ND | A | B1 | H1 |
| Mi2 | *M. guilliermondii* | Strawberry jam | Bubbles | | | A | B1 | H1 |
| Mi3 | *M. guilliermondii* | Strawberry jam | Bubbles | $3.58 \times 10^7$ | $1.28 \times 10^7$ | A | B1 | H1 |
| Mi4 | *M. guilliermondii* | Strawberry yogurt | Swollen | $6.03 \times 10^7$ | $1.30 \times 10^7$ | A | B1 | H1 |
| Mi5 | *M. guilliermondii* | Strawberry yogurt | Swollen | | | A | B1 | H1 |
| Mi6 | *M. guilliermondii* | Strawberry yogurt | Swollen | $3.43 \times 10^7$ | $8.24 \times 10^6$ | A | B1 | H1 |
| Mi7 | *M. guilliermondii* | Berry yogurt | Swollen | $3.53 \times 10^7$ | $9.27 \times 10^6$ | A | B1 | H1 |
| Mi8 | *M. guilliermondii* | Berry yogurt | Swollen | $1.52 \times 10^8$ | $5.37 \times 10^6$ | A | B1 | H1 |
| YN2 | *M. guilliermondii* | Plain yogurt | Not swollen | $6.52 \times 10^6$ | $1.99 \times 10^6$ | A | B1 | H1 |
| YN5 | *M. guilliermondii* | Plain yogurt | Not swollen | $2.47 \times 10^7$ | $2.31 \times 10^6$ | A | B1 | H1 |
| YN8 | *M. guilliermondii* | Plain yogurt | Not swollen | $1.40 \times 10^8$ | $8.88 \times 10^6$ | A | B1 | H1 |
| YF6 | *M. guilliermondii* | Strawberry yogurt | Swollen | $9.72 \times 10^7$ | $6.57 \times 10^6$ | A | B1 | H1 |
| YF9 | *M. guilliermondii* | Strawberry yogurt | Swollen | $1.15 \times 10^8$ | $1.63 \times 10^7$ | A | B1 | H1 |
| YFB1 | *M. guilliermondii* | Berry yogurt | Swollen | $1.26 \times 10^8$ | $3.16 \times 10^6$ | A | B1 | H1 |
| YFB4 | *M. guilliermondii* | Berry yogurt | Swollen | $5.80 \times 10^7$ | $6.16 \times 10^6$ | A | B1 | H1 |
| YFB7 | *M. guilliermondii* | Berry yogurt | Swollen, bubbles | $1.14 \times 10^8$ | $3.85 \times 10^6$ | A | B1 | H1 |
| YA10 | *M. guilliermondii* | Apricot yogurt | Swollen, bubbles | $1.19 \times 10^8$ | $1.45 \times 10^7$ | A | B1 | H1 |
| MA11.1 | *M. guilliermondii* | Apricot jam | Bubbles | $4.60 \times 10^6$ | $2.38 \times 10^5$ | A | B1 | H1 |
| MA11.2 | *W. anomalus* | Apricot jam | | | | - | - | - |
| **Strains from: Spanish type culture collection** | | | | | | | | |
| CECT 1456 [T] | *M. guilliermondii* | Insect frass on *Ulmus americana* | | - | - | B | B2 | H1 |
| CECT 1019 | *M. guilliermondii* | Flower of *Gentiana imbricata* | | - | - | F | B1 | H2 |

**Table.1.** *Cont.*

| Isolated Strains | Identification [a] | Origin | Spoilage | CFU/g | Standard Deviation (Std. dev.) | RFLPs mtDNA | RFELP IGS | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *Hinf*I | *Hha* I (B) | *Hae* III (H) |
| **Strains from: Spanish type culture collection** | | | | | | | | |
| CECT 1438 | *M. guilliermondii* | Pozol, Mexican fermented maize dough | - | - | - | A | B1 | H2 |
| CECT 10157 | *M. guilliermondii* | Fruit in syrup | - | - | - | C | B1 | H2 |
| CECT 1021 | *M. guilliermondii* | Soil from drilling care | - | - | - | D | B3 | H2 |
| CECT 12791 | *M. guilliermondii* | Soil | - | - | - | D | B1 | H2 |
| CECT 12839 | *M. guilliermondii* | Beer var. garrafal | - | - | - | E | B1 | H2 |

Notes: *M.* and *W.* correspond to the genus *Meyerozyma* and *Wickerhamomyces,* respectively. ND: Not Determined. [a] All isolates were identified by molecular methods (RFLP of 5.8S-ITS with *Hinf*I, *Hha*I, and *Hae*III endonucleases). *M. guilliermondii* strain. Identification was subsequently confirmed by *Taq*I-5.8S-ITS [14]; also, traditional morpho-physiological identification was performed [16,17].

**Table 2.** Some physiological characteristics of the *M. guilliermondii* strains studied in this work.

| | Isolated Strains | Type Strain [a] |
|---|---|---|
| **Assimilation-Growth** | | |
| D-Glucose | + | (+, D) |
| D-Galactose | + | (−, D) |
| Sucrose | + | (+, D) |
| Lactose | − | − |
| DL-Lactate | + | − |
| **Fermentation** | | |
| D-Glucose | + | + |
| D-Galactose | + | W |
| Sucrose | + | + |
| Lactose | − | − |

Notes: [a] Data from Barnett *et al.* [16]. +, growth within 7 days; −, no growth after 14 days; W, weak growth response, D, delayed growth.

*2.6. Assessment of Gas Production in Lab-Contaminated Organic Yogurt*

Gas production was followed as described by Casas *et al.* [24]. The study included two types of controls, one in which the yogurt samples were pasteurized in order to inactivate Lactic Acid Bacteria (LAB) and a second one, a pasteurized, non-inoculated plain yogurt. Samples were inoculated with two different charges of inocula (i) to reach an initial population of about 4 (low) or (ii) 400 (high) CFU/g (Table 3). The inoculated yogurts were incubated at 8 °C or 28 °C. Growth was measured as CFU/g. Duplicate samples were analyzed once a week for one month.

**Table 3.** Artificial lab-inoculated yogurts, final CFU/g and gas produced in yogurts with low (4 CFU/g) and high (400 CFU/g) inocula after incubation at two temperatures.

| Yoghurt Type | Inoculum | Incubation Temperature | | | | | |
|---|---|---|---|---|---|---|---|
| | | 8 °C | | | 28 °C | | |
| | | CFU/g | Std. dev. | Gas | CFU/g | Std. dev. | Gas |
| **Plain** | Low | $4.30 \times 10^6$ | $4.24 \times 10^5$ | No | $4.28 \times 10^6$ | $4.83 \times 10^5$ | No |
| | High | $5.41 \times 10^6$ | $5.55 \times 10^5$ | No | $1.56 \times 10^6$ | $3.44 \times 10^5$ | No |
| **Past. plain** | Low | $2.99 \times 10^6$ | $2.40 \times 10^5$ | No | $1.00 \times 10^7$ | $5.85 \times 10^5$ | No |
| | High | $9.29 \times 10^6$ | $1.33 \times 10^6$ | No | $1.24 \times 10^7$ | $1.36 \times 10^5$ | No |
| **Fruit** | Low | $1.21 \times 10^7$ | $3.12 \times 10^5$ | Low | $5.32 \times 10^6$ | $3.92 \times 10^5$ | High |
| | High | $8.56 \times 10^6$ | $8.97 \times 10^5$ | Low | $5.52 \times 10^6$ | $8.91 \times 10^5$ | High |
| **Past. fruit** | Low | $2.96 \times 10^7$ | $5.43 \times 10^6$ | Low | $9.32 \times 10^6$ | $7.55 \times 10^5$ | High |
| | High | $5.57 \times 10^6$ | $1.32 \times 10^6$ | Low | $1.08 \times 10^7$ | $1.10 \times 10^6$ | High |

Notes: Past.: pasteurized. No: No gas observed. All experiments included a minimum of at least two independent replicates. Pasteurized yoghurt was used as a control.

## 3. Results

*3.1. Strain Isolation and Identification*

Samples of organic plain, strawberry, berry, and apricot yogurt as well as apricot jam, were analyzed, as described in the Materials and Methods section, to quantify the contamination. The results are shown in Table 1. In all the cases a high number of yeast colonies, $10^7$–$10^8$ CFU/g, were isolated. Yeast colonies were identified as described in the corresponding section. All the strains, except one, belonged to the species *Meyerozyma guilliermondii*. The strain MA11.2 was identified as *Wickerhamomyces anomalus* (formerly *Pichia anomala*). A coincidence of 100% was obtained by both methods used. The amplified ITS region presented an identical size for all the strains (620 bp) but the restriction profile was different. All *Meyerozyma* strains had the same restriction profile (size in bp, *Hha*I: 290 + 270, *Hae*III: 390 + 120 + 80, and *Hinf* I: 320 + 290) meanwhile *Wickerhamomyces anomalus* strain presented another one (*Hha*I: 570, *Hae*III: 620, and *Hinf* I: 310 + 310). The suitability of the identification of *M. guilliermondii* strains was confirmed by the *Taq*I-5.8-ITS method described by Romi *et al*. [15]. This method allows us to distinguish between the closely related *M. guilliermondii* and *M. caribbica*. All the strains used in this work were confirmed as *M. guilliermondii*. In addition, two selected strains have been confirmed by sequencing the 5.8S ITS rDNA region. The sequences were compared with the type strain and obtained the same identification.

It is noteworthy that among the results of the physiological tests (Table 2) none of the *Meyerozyma* isolates were able to ferment lactose but all of them fermented glucose, sucrose, and, more weakly, galactose. Moreover, all were able to grow on lactate. These traits, relevant to the spoilage problem, prompted us to analyze the physiological characteristics of this lactate-positive group that allow it to contaminate, grow heavily, and in some cases even spoil fermented dairy products.

*3.2. Comparison of Typing Techniques*

Two different typing methods previously used for yeasts were evaluated as described in the Materials and Methods section. The best discriminatory power was achieved by mtDNA-RFLP when compared with PCR-RFLP of the IGS region of rDNA. As can be seen in Table 1, *Hinf* I-mtDNA-RFLP produced seven different restriction patterns (A to F). All the lactate positive strains of *M. guilliermondii* tested, including those isolated from yogurt and jams as well as the other lactate-positive collection strain CECT 1438 (CBS 6557) isolated from maize lactic fermentation, exhibited the same restriction pattern with mtDNA-RFLP (pattern A). This pattern differentiates the lactate-positive strains from the others, such as, for example, the soil strains that were grouped in pattern D. The IGS-PCR RFLP method produced three different patterns with the endonuclease *Hha*I (B1–B3) and two with the endonuclease *Hap*II (H1 and H2). Bringing both methods together, mtDNA-RFLP and IGS-PCR RFLP, it was observed that all strains isolated in this work from the industry present the same pattern of AB1H1; meanwhile, the rest of the strains included in this study presented different ones. Moreover, each one of the strains studied in this work presented its own pattern while applying both methods.

*3.3. Analysis of the Survival, Growth, and Spoiling Abilities of the Strains*

As the identification studies had shown that the strains were unable to neither grow nor ferment lactose, growth and fermentation were tested with other carbon sources available in yogurt, lactate and galactose from milk, and sucrose from jams. The results are shown in Figures 1 and 2. All those carbon sources were able to support growth, producing a similar concentration of yeast (about $10^7$ CFU/g) to that found in natural (Table 1) and lab-contaminated (Table 3) yogurt. Alcoholic fermentation was followed quantitatively by ethanol production after seven days' incubation (see Materials and Methods section). It was found that the spoilage strains fermented sucrose and had a weak fermentation of galactose, which was even weaker in the presence of lactate. However, the fermentative capacities were far below that of *S. cerevisiae* used as control (Figure 2). To prove that *M. guilliermondii* was responsible for the spoilage, several samples of fruit and plain organic yogurt from the same brand, bought in a supermarket, were inoculated with low (4 CFU/g ) or high inocula (400 CFU/g). The assay was performed on yogurts with or without active lactic bacteria, as described in Materials and Methods. After a week of incubation at 28 °C (a temperature easily reached in Spain in spring or summer), a stationary population of about $10^7$ CFU/g was observed, similar to that found in the original spoiled yogurts, regardless of the type of yogurt or the amount of inocula. No statistically significant differences were found between the pasteurized or non-pasteurized samples (see Table 3), indicating that, even with a low inoculum, there was not any inhibitory effect from lactic bacteria on yeast growth. Gas production was detected after the first week, but, as expected, only in yogurts containing fruit or jam (*i.e.*, with added sugars) and not in plain yogurt, in which lactose was the only fermentable source. Incubation at

low temperatures (8 °C) decreased the growth rate but not the yield because the same final population was obtained, although eight days later (Table 3). A remarkable result obtained in this experiment was the uncoupling of growth and fermentation observed at 8 °C, a temperature at which fermentation, but not growth, was strongly inhibited. No gas was detected at 8 °C after 11 days of incubation, although the yeast population had grown above of $10^6$ CFU/g. Only after 35 days were small amounts of gas detected, again only in yogurts with fruit (Table 2).

Besides the tolerance to pH (good growth on lactate, Figure 1) and temperature (growth at 8 ºC, Table 3) the isolated strains also showed a high osmotolerance, because they were able to grow in jams (0.80 aw (water activity)) with a high yield ($4.6 \times 10^6$ CFU/g, see Table 1).

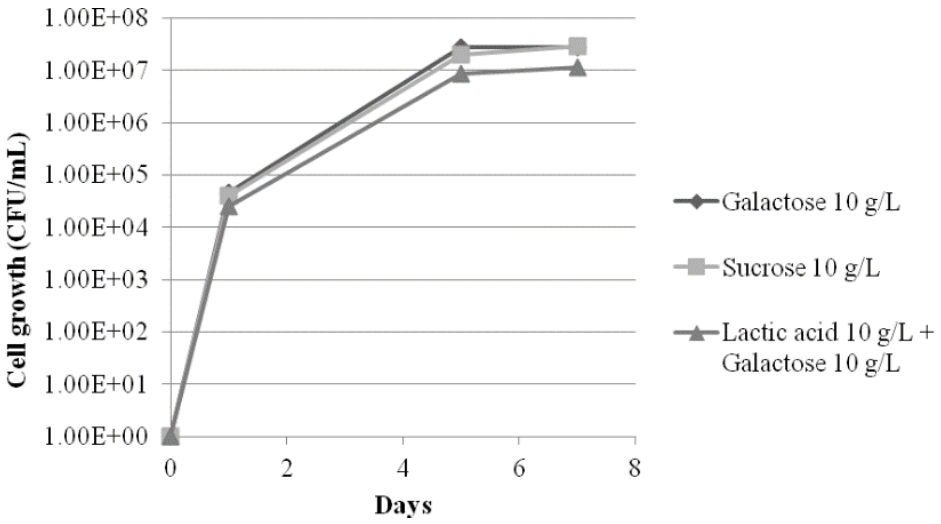


**Figure 1.** Growth kinetics of one isolated strain (Mi4) in three different carbon sources.

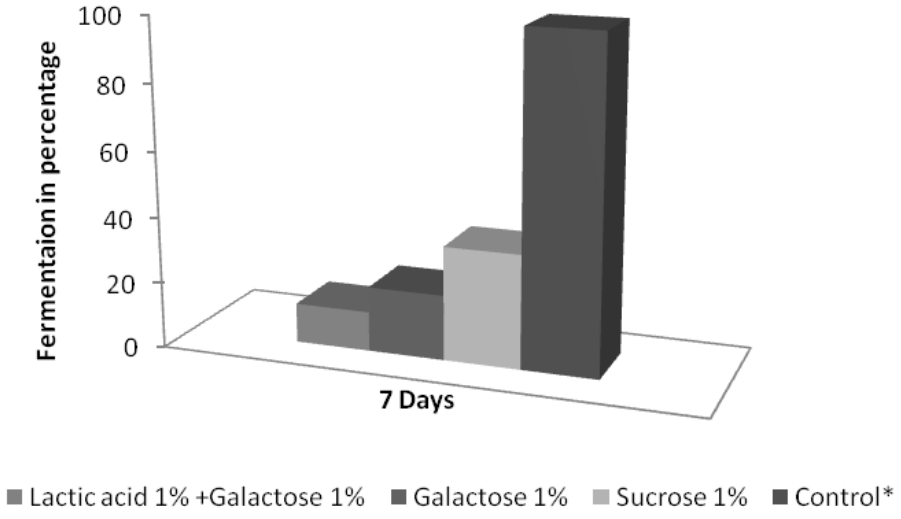


**Figure 2.** Sugar fermentation capacities of one isolated strain (Mi4) after seven days, measured as ethanol production and expressed as % of the control strain*: *Saccharomyces cerevisiae* growing on glucose, 1%.

## 4. Discussion

The recent distribution of the Q-9 strains of *Pichia* in five new genera as well as the previous recognized misidentifications as *Debaryomyces hansenii* [25] have introduced some uncertainty about *Meyerozyma (Pichia) guilliermondii* ecology. In this work we describe the presence of molecularly identified *M. guilliermondii* in yogurt. It is possible that some of the *Debaryomyces hansenii* strains previously isolated and morpho-physiologicaly identified from yogurt [26] could have been *M. guilliermondii* strains. Microbial spoilage is a very complex process. It is the final result of many intrinsic, extrinsic, implicit, and industrial factors that interact through a complex network of relationships [26]. In many traditional food processes, the relative influence of the different factors have been empirically equilibrated, thereby preventing spoilage. However, changes designed to improve the product, the production process, and/or to follow the tendencies of the market, may introduce such strong alterations in the interaction network that the risk of spoilage increases. To prevent spoilage, yogurts include preservatives such as sorbate. However, the organic yogurts studied in this work do not contain preservatives. The first surprise in this study was to find that, in contrast to the widespread assumption that gas spoilage in dairy products is caused by a relatively narrow group of lactose fermenting yeasts [27], the responsible microorganism was *M. guilliermondii.* This species is unable to ferment lactose and only weakly ferments other sugars [2]. All the Koch postulates were demonstrated in this case: the microorganism was found in all the samples; and it was isolated in pure culture, identified, typed, and re-inoculated to produce the same effect (Tables 1 and 3). As it cannot ferment lactose, gas spoilage was only produced in those yogurts in which a fermentable carbon source had been provided by the addition of jam. The isolated strains are poor fermenters when compared with *S. cerevisiae* (Figure 2), but their growth in the yogurts was so dense that the gas produced was enough to swell the yogurt packages (Tables 1 and 2). The similar results on yeast growth (Table 2) obtained with yogurts with or without lactic bacteria, after pasteurization, shows that there is no competition for nutrients between yeast and bacteria nor any other type of LAB inhibition on yeast growth. This association between lactate-positive yeasts and LAB could be a good example of synergistic interaction between both microorganisms, with LAB producing the carbon source for yeasts and they, in return, decreasing the inhibitory effect of acid pH by consuming lactic acid [28]. Moreover, this study also shows that, at least in this species, yeast fermentation and growth are not tightly coupled and can easily be dissociated, as occurs at low temperatures. With incubation at 8 °C, growth is only retarded but fermentation is almost completely inhibited (Table 3). This could be explained by the fact that fermentation is not the main source of ATP for this strain, which is able to oxidize lactate and, consequently, growth can proceed without fermentation. Our present hypothesis is that growth and spoilage are relatively separate processes in this and other cases, when the responsible microorganism is oxidative. A strong growth would take place with lactate (as the only carbon and energy source) and when oxygen, but not sugar, was exhausted, sugar fermentation would occur, producing spoilage but no significant growth.

With respect to the typing methodologies, our results, in agreement with Martorell *et al.* [29], show that concerning *M. guilliermondii*, mtDNA restriction with *Hinf*I provided high variability among the strains. This variability seems to bring to light physiological characteristics, as revealed by the fact that all strains with pattern A are involved in lactic fermentation and all strains with pattern D have been isolated from soil (Table 1). On the other hand, PCR-RFLP of IGS is a technique that has been recently

demonstrated as an efficient typing method for the discrimination of strains belonging to several species of the *Zygosaccharomyces* genus [30]. However, although this technique produces more clear restriction patterns than RFLP-mtDNA and equally reproducible results, by itself it constitutes a typing method with a minor power of discrimination for *M. guilliermondii*. The mtDNA-RFLP method properly identifies lactate-positive *M. guilliermondii* strains (pattern A), but a combination of both methods is necessary for obtaining 100% discrimination between strains (Table 1). From the typing studies we can hypothesize that the yeasts possibly reached the factory via contaminated jams. Subsequently, they colonized the equipment, and this would have extended to contaminate plain yogurt. As heat tolerance in yeast is higher in high-sugar foods [31], if present in the fruit, they were able to survive the heat treatment during jam production. On the other hand, the combination of techniques for typing and its suitability in clinical strains may be interesting. The fungemia caused by Candida (*Meyerozyma) guilliermondii* has increased in recent years [32]. The health significance of yeast in foods has been considered to be minimal, if not negligible, because pathogenic yeasts, such as *Candida albicans* or *Criptococcus neoformans*, are not transmitted through foods [33]. However, it may be necessary to revise this criterion, especially in relation to organic foods, where yeasts can reach high densities. These dense populations may be involved in the development of adverse responses in humans, such as allergies [34]. Corte *et al*. [35], when analyzing the question of whether isolates from different sources are physiological and genetically similar, found differences by using the Fourier transform infrared spectroscopy **(**FTIR) technique between fruit and environmental isolates, but none of the tools employed permitted us to distinguish between medical and environmental isolates, a very important factor to take into account in future research.

## 5. Conclusions

We have proven that the gas spoilage of the analyzed organic yogurts was due to the fermentation of the fruit and jam sugars by a *M. guilliermondii* type A strain (mtDNA typing). This strain grows up to high counts ($10^6$–$10^7$ CFU/g) in yogurt, using lactate as its carbon source. In the presence of fermentable sugars, fermentation was almost completely inhibited at 8 °C, which points to the relevance of temperature in controlling spoilage. However, at this low temperature, yeast growth continues, although at a slower rate, reaching a similar maximum population as at 28 °C, but a few days later.

Yeasts such as those studied in this research should be controlled throughout the whole production process from organic agriculture to the final product, for quality and safety reasons, especially considering consumers with compromised immunity.

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## Author Contributions

Petra Wrent, María-Isabel de Silóniz and José M. Peinado conceived and designed the experiments; Petra Wrent and Eva-María Rivas performed the experiments; Petra Wrent, María-Isabel de Silóniz and José M. Peinado analyzed the data; Elena Gil de Prado contributed reagents/materials/analysis tools; Petra Wrent and María-Isabel de Silóniz wrote the paper.

## Conflicts of Interest

The authors declare no conflict of interest.

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# Capítulo 7

**Discusión General**

La mayoría del conocimiento acumulado sobre las levaduras hace referencia a *S. cerevisiae*, cuya utilidad industrial es sobradamente conocida y los estudios sobre su fisiología y genoma son frecuentes (Souciet *et al.*, 2009). No obstante, gracias a sus características fisiológicas y genéticas otras levaduras colonizan y sobreviven en diversos tipos de alimentos o bebidas (Stratford, 2006). Es más, las nuevas o modificadas formulaciones de los alimentos, en algunas ocasiones, favorecen su desarrollo (Deák, 2008). Según la especie y el alimento, las levaduras pueden contribuir a mejorar las características organolépticas de los productos en los que se desarrollan o, como es habitual, los deterioran (Stratford, 2006).

El género *Zygosaccharomyces*, en términos de su capacidad deteriorante se considera uno de los más peligrosos por su osmotolerancia, capacidad fermentativa y resistencia a algunos conservantes (Casas *et al.*, 2004; Stratford, 2006) e incluye especies como *Z. rouxii* ampliamente reconocidas como deteriorantes. Sin embargo, y a pesar de lo que acabamos de comentar, su resistencia al estrés hace que sea una especie atractiva para la producción industrial de vinagre balsámico y de alimentos orientales como el miso (Solieri y Giudici, 2008; Sujaya *et al.*, 2003).

De igual modo, dentro del género *Debaryomyces*, la especie halotolerante *D. hansenii* destaca tanto por su interés básico como aplicado, como se ha comentado en el capítulo 1 (Introducción y Objetivos) (Andrade *et al.*, 2014; Cano-Garcia *et al.*, 2013; Cruz *et al.*, 2000; Liu y Tsao, 2009; Martín *et al.*, 2006; Padilla *et al.*, 2014). A pesar de los aspectos beneficiosos, en nuestro laboratorio hemos podido comprobar que bajo ciertas circunstancias *D. hansenii* es capaz de

deteriorar los alimentos en los que se encuentra (Quirós, 2005). Esto es posible porque aunque se considera una especie oxidativa (Gancedo y Serrano, 1989), fermenta los azúcares y, por consiguiente, puede hinchar los envases produciendo el rechazo del consumidor (Quirós, 2005; Quirós *et al.*, 2008).

Por otro lado, *M. guilliermondii* es una especie cuya ecología actualmente está poco clara debido a los frecuentes errores de identificación que se cometen con algunos métodos tradicionales, como ya hemos comentado (Desnos-Ollivier *et al.*, 2008; Kim *et al.*, 2014). El hecho de que *M. guilliermondii* (teleomorfo de *C. guilliermondii*) aparezca como contaminante en alimentos tiene una relevancia más allá de los problemas de pérdidas económicas que pudieran derivar de ello, puesto que está especie parece estar implicada entre el 1 y 2% de todas las candidemias diagnosticadas (Pfaller *et al.*, 2006). La relevancia de las levaduras en la salubridad de los alimentos suele considerarse despreciable porque las levaduras patógenas oportunistas como *C. albicans* o *C. neoformans* no se transmiten a través de los alimentos (Fleet, 1992). Sin embargo, este criterio, en nuestra opinión, necesita revisión ya que se han descrito alergias o respuestas adversas en humanos debidos a la presencia de levaduras (Fleet y Balia, 2006).

Esta Tesis persigue el desarrollo de herramientas moleculares, que se puedan aplicar en los propios laboratorios de las industrias, para la detección (*D. hansenii,* híbridos de *Zygosaccharomyces*) o bien la tipificación de cepas (*Z. rouxii* y *M. guilliermondii*). La tipificación de cepas es un proceso que se aplica después de la identificación y puede buscar diferentes objetivos. Por un lado seleccionar las cepas más idóneas para una actividad determinada, el método debe discriminar entre biotipos con

propiedades diferentes que puedan mejorar las características de un producto como, por ejemplo, la capacidad fermentativa o la producción de compuestos volátiles (Andrade *et al.*, 2009; Martorell *et al.*, 2005; Suezawa *et al.*, 2008). Por otro lado, el objetivo puede ser diferenciar cada una de las cepas. Nosotros en este trabajo hemos seguido este segundo abordaje, eligiendo aquella técnica que proporcione el mayor grado de discriminación entre cepas. En el contexto del deterioro de alimentos el método que logra el mayor grado de discriminación permitirá "trazar" dicha cepa a lo largo de la cadena de producción, proporcionando un arma incluso para posibles demandas legales si es necesario (Wrent *et al.*, 2010).

Para conseguir nuestros objetivos, en esta Tesis hemos tenido que probar diferentes métodos hasta obtener la especificidad, fiabilidad y exactitud necesaria en cada caso, ya que no existe un método universal que además sea fácil de aplicar en las industrias. Como ya hemos comentado, hoy en día la identificación tradicional ha dado paso a las técnicas moleculares. Entre estas últimas, constituye una práctica habitual en los laboratorios, sobre todo cuando se trata de grandes colecciones de cepas aisladas de alimentos, la utilización de los perfiles obtenidos mediante la restricción con tres endonucleasas de la región 5,8S-ITS del DNA ribosómico y posterior comprobación sólo de algunas cepas, mediante la secuenciación del dominio D1/D2 del gen 26S del rDNA o de la región 5,8S-ITS del rDNA, dentro de las cepas que presentan el mismo patrón. Se da la circunstancia de que, basándose en diferencias genéticas (Corredor *et al.*, 2000; Groenewald *et al.*, 2008; Prillinger *et al.*, 1999; Quirós *et al.*, 2006), la última revisión taxonómica restauró como nuevas especies del género a las especies *D. fabryi* y *D. subglobosus* (Suzuki *et al.*,

2011). Es de destacar, que la técnica RFLP 5,8S-ITS rDNA que acabamos de comentar y que es muy utilizada rutinariamente para la identificación de levaduras, por la facilidad que supone la base de datos de la CECT (http://www.yeast-id.org/), produce perfiles idénticos para las especies *D. hansenii*, *D. fabryi* y *D. subglobosus* (Fig. 2, Capitulo 4). Además, la base de datos de la CECT no se encuentra actualizada en función de las reclasificaciones descritas para varias levaduras (Kurtzman *et al.*, 2011a). Por otro lado, la secuencia del dominio D1/D2 del gen 26S del rDNA y la región 5,8S-ITS del rDNA nos permite identificar especies del género *Zygosaccharomyces*, pero no detecta posibles híbridos dentro de la especie *Z. rouxii*. Mientras que en *Meyerozyma*, la secuencia del dominio D1/D2 del gen 26S del rDNA no aporta una identificación clara de las dos especies de este género (*M. caribbica* y *M. guilliermondii*) ni las diferencia de otras especies del *cluster* de *Meyerozyma* como *C. carpophila* (Romi *et al*., 2014). Del mismo modo, la técnica IGS-PCR RFLP del rDNA descrita por Quirós *et al*. (2006) separa especies dentro del género *Debaryomyces*, pero para *Zygosaccharomyces* nos ha resultado de utilidad como técnica de tipificación en especies como *Z. bailii*, *Z. mellis* y *Z. rouxii*. Sin embargo, esta técnica en *Meyerozyma* no consigue, con las enzimas ensayadas (*Hae*III, *Hap*II, *Hha*I y *Mbo*I) diferenciar entre cepas.

A continuación realizamos una discusión más detallada

**_Z. rouxii_ desarrollo de un método de tipificación y estudio de las cepas CECT 11923 y CECT10425 (Wrent _et al._, 2010; Wrent _et al._, 2015c)**

Para desarrollar el método de tipificación de cepas, partimos de la hipótesis de que la región IGS, por su variabilidad (Sugita *et al.*, 2001)

podría ser una diana adecuada. Con el objetivo de amplificar fragmentos de un tamaño superior a 6000 pb, en este trabajo hemos modificado el protocolo previamente desarrollado para otras especies en nuestro laboratorio por (Quirós *et al.*, 2006; Romero *et al.*, 2005). El método que proponemos (Wrent *et al.*, 2010) está basado en la amplificación de esta región con los cebadores CNL12 (5'CTGAACGCCTCTAAGTCAG3') y CNS1 (5'GAGACAAGCATATGACTACTG3´) y posterior digestión con tres endonucleasas (*Hap*I, *Hha*I y *Mbo*I). Este método, al contrario que otros estudios descritos para la tipificación de cepas de esta especie (Martorell *et al.*, 2005), no requiere de la combinación de dos técnicas diferentes.

Como puede verse en la Tabla 1 (capítulo 2), el estudio se llevó a cabo con alrededor de 60 cepas correspondientes a las diferentes especies del género *Zygosaccharomyces* reconocidas hasta ese momento (Wrent *et al.*, 2010). Actualmente la restructuración taxonómica mencionada en el capítulo 1 (James y Stratford, 2011), reduce el género a seis especies (*Z. bailii*, *Z. bisporus*, *Z. kombuchaensis*, *Z. lentus*, *Z. mellis* y *Z. rouxii*). Es destacable que analizando conjuntamente los patrones obtenidos con las tres endonucleasas utilizadas, se obtiene un 100% de variabilidad en cada una de las cepas de las especies *Z. mellis* (10 cepas) y *Z. rouxii* (33 cepas) y del 70% para *Z. bailii* (7 cepas). Esta variabilidad es superior a la encontrada por otros autores utilizando otros métodos como RFPL del mtDNA, RAPD`s o análisis de las secuencias de la región 5,8S ITS del rDNA y del dominio D1/D2 del gen 26S del rDNA (Martorell *et al.*, 2005; Suezawa *et al.*, 2008). No obstante, consideramos que en el caso de *Z. bailii* posiblemente sea superior, puesto que como ya se ha comentado en la discusión del capítulo 2, nuestra hipótesis es que las cepas que presentan el mismo patrón de restricción puedan ser la misma cepa. Este sería el

caso de las cepas *Z. bailii* May (12) y May (13) o de CECT 1924 y CECT 1898$^{T}$, tanto en el primer caso como en el segundo las cepas fueron aisladas en el mismo estudio, incluso en el primer ejemplo de la misma muestra (Tabla 1, capítulo 2). Es de destacar que el método que hemos desarrollado nos ha permitido detectar cepas mal identificas (*Z. mellis* CBS711 y CBS 7412) con perfiles de restricción que se agrupaban dentro del *cluster* de *Z. rouxii* (Fig. 3, capitulo 2) y que presentaban para cada enzima las bandas características de esta especie que se muestran a continuación.

| | |
|---|---|
| *Hap*II | 970+700+400+300+160 |
| *Hha*I | 960+700+450 |
| *Mbo*I | 760+410+350+260+170 |

Efectivamente, el análisis de las secuencias del dominio D1/D2 del gen 26S del rDNA y de la región 5,8S-ITS rDNA confirmaron que se trataba de dos cepas de *Z. rouxii* erróneamente identificadas por métodos morfo-fisiológicos como *Z. mellis*.

Por otro lado, éste método puso de manifiesto que las cepas de *Z. rouxii* CECT 11923 y CECT 10425 presentaban diferencias importantes en el patrón de restricción que nos hicieron dudar de su correcta identificación (Tablas 1 y 2, capítulo 2) a pesar de que el tamaño del fragmento amplificado de la región IGS correspondía al tamaño estimado para *Z. rouxii* (4200 pb) y la secuenciación de la región 5,8S-ITS del rDNA y del dominio D1/D2 del gen 26S del rDNA confirmaron dicha identificación (Wrent *et al.*, 2010). El dendograma construido mediante el análisis de los patrones de restricción de la región IGS (Fig.3, capítulo 2) agrupaba estas

cepas con los híbridos de *Zygosaccharomyces* (NCYC 1682, NCYC 3060 y NCYC 3061) previamente descritos por (James *et al.*, 2005). Este autor aún reconociendo la indudable utilidad de la secuenciación en la identificación de especies cuestionó su validez para la identificación de especies híbridas cuando se aplica a un único gen.

La hibridación entre una o diferentes especies es un hecho natural que en algunas ocasiones produce especímenes viables (Groth *et al.*, 1999) y que se supone que juega un papel importante en la evolución (Wu *et al.*, 2008). Las cepas híbridas de *Saccharomyces* aisladas de vino son un buen ejemplo del interés y la importancia que los híbridos despiertan (Borsting *et al.*, 1997; Casaregola *et al.*, 2001; de Barros Lopes *et al.*, 2002; Nguyen *et al.*, 2000; Peris *et al.*, 2012). Desde la antigüedad *S. cerevisiae* y las especies relacionadas, que pertenecen al grupo de *Saccharomyces sensu stricto,* son los cultivos iniciadores más importantes para la producción de alimentos y bebidas fermentadas. En los procesos de vinificación los híbridos naturales o artificiales aportan una complejidad sensorial al vino y las hacen más resistentes a las fluctuaciones ambientales que sus especies parentales (Sipiczki, 2008; Solieri *et al.*, 2015). Algunos autores consideran que los híbridos naturales de *Zygosaccharomyces* pueden ser más frecuentes de lo que se piensa (James *et al.*, 2005; James y Stratford, 2011). Por lo tanto, una identificación precisa de los mismos es muy deseable, no sólo desde el punto de vista taxonómico sino también práctico. Existen en las Colecciones de Cultivos Tipo especies que se utilizan como referencia en diversos estudios, por ejemplo genéticos (*Z. rouxii* ATCC 42981) cuyos resultados se extrapolan a toda la especie y que, posteriormente, se ha demostrado que son el resultado de la hibridación (Gordon y Wolfe, 2008). Por otro lado, dado

que la poliploidización puede mejorar la capacidad de soportar condiciones estresantes (Piotrowski *et al.*, 2012), los híbridos son buscados por la industria. De hecho, actualmente se sabe que los híbridos de *Zygosaccharomyces* juegan un papel importante en las características finales de algunos productos de la cocina asiática, ahora universales, como la salsa de soja y la pasta de miso (Suezawa *et al.*, 2008; Sujaya *et al.*, 2003; Tanaka *et al.*, 2012). Por ello se han generado híbridos artificiales de *Zygosaccharomyces* en el laboratorio con el fin de mejorar el sabor del producto final (van der Sluis *et al.*, 2001).

Como hemos comentado anteriormente a la vista de los resultados obtenidos con las cepas de *Z. rouxii* CECT 11923 (CBS 4021) y CECT 10425, en este trabajo partimos de la hipótesis de que se trataba de cepas híbridas. La primera fue aislada por Onishi y depositada en la CBS (CBS 4021) como *S. acidifaciens* var. *halomembranis*, aunque más tarde fue reclasificada por Kurtzman como *Z. rouxii* (datos de la CBS), mientras que la cepa CECT 10425 fue aislada de miel, identificada por Santa-María y depositada en 1991 en la CECT (datos de la CECT). Con el objetivo de comprobar esta hipótesis analizamos los resultados obtenidos a) con los cebadores específicos desarrollados por Harrison *et al*.(2011) para la identificación de especies del género *Zygosaccharomyces*, b) el número de copias de la región 5,8S-ITS y la secuencia de las mismas, c) el número de copias de los genes *SOD2* e *HIS3* (James *et al.*, 2005); además, d) del polimorfismo de la región IGS1. Pudimos comprobar que la cepa CECT 11923 puede ser un híbrido entre *Z. rouxii* y *Z. pseudorouxii* puesto que posee dos copias de los genes *SOD2* e *HIS3* (Tabla 3, capitulo 3) y cada uno de los pares de cebadores específicos para *Z. rouxii* y *Z. pseudorouxii* (Harrison *et al.*, 2011) dieron lugar a clarísimos productos de amplificación

(Tabla 4, capitulo 3). No obstante, debemos señalar que este autor no desarrolló cebadores específicos para *Z. sapae.* La secuencia de una de las copias de la región 5,8S-ITS que arbitrariamente hemos denominado D presentó una identidad del 100% con la secuencia de esta región en la cepa tipo de *Z. rouxii* (CECT1232$^{T}$) mientras que otras entre el 97 y el 99% con una cepa descrita recientemente como coespecífica de *Z. sapae*. *Z. sapae* constituye una nueva especie aislada de vinagre balsámico que al igual que la cepa CECT 11923 no fermenta maltosa y presenta muchas similitudes con *Z. pseudorouxii*. De ésta última, sólo se conoce una cepa y no ha sido descrito formalmente por lo que sería un *nomen invalidum* (James *et al.*, 2005; Solieri *et al.*, 2013). Con respecto a la cepa CECT 10425 mantenemos la hipótesis de que pueda tratarse de una cepa híbrida entre Z*. pseudorouxii* y *Z. sapae*. No obstante su asignación es menos clara, presenta dos copias del gen *HIS3* una de ellas correspondiente a *Z. rouxii* mientras la otra corresponde a *Z. pseudorouxii.* Posee una única copia del gen ATCC 42981 *Z-SOD22* y hemos podido describir tres copias de la región 5,8S-ITS que presentaban una identidad del 99% con la secuencia de esta región para una cepa coespecífica con *Z. sapae* (*Z. rouxii* NBRC10669) (Tabla 5, capitulo 3) (Wrent *et al.*, 2015c).

Durante el desarrollo de esta parte del trabajo hemos diseñado, basándonos en el polimorfismo de la región IGS1, unos cebadores específicos para el reconocimiento de híbridos de *Zygosaccharomyces* (HibZF/HibZR) (Wrent *et al.*, 2015c). En ambos casos, tanto en la cepa CECT 11923 como en la cepa CECT 10425, se obtuvo un claro producto de amplificación, sugiriendo nuevamente que se trata de especies híbridas. En el caso de la cepa CECT 11923 del mismo tamaño (700 pb) que el

obtenido en las cepas híbridas descritas por James *et al*.(2005) (NCYC 1682, NCYC 3060 and NCYC 3061), mientras en la cepa CECT 10425 fue de 500 pb. Se obtuvieron productos de amplificación en todas las cepas híbridas ensayadas pero no en el resto de cepas pertenecientes a diferentes especies de levaduras (Tabla 1, capitulo 3). Para *S. cerevisiae* se han propuesto diferentes metodologías para discriminar entre las especies parentales y los híbridos, tales como el cariotipo (Giudici *et al.*, 1998; Le Jeune *et al.*, 2007), el análisis de microsatélites (Erny *et al.*, 2012), secuenciación de regiones divergentes del rRNA, marcadores de genes constitutivos (Gancedo y Serrano, 1989; Gonzalez *et al.*, 2006) o estrategias que implican dos etapas, la primera una amplificación de la región ITS 1 ó 2 seguida de la restricción con endonucleasas diagnósticas y una segunda con la utilización de minisatélites intragénicos (Solieri *et al.*, 2015). Sin embargo, para los híbridos de *Zygosaccharomyces* no se había propuesto, hasta ahora, ningún método específico de detección. El método de PCR que hemos desarrollado, además de fiable y reproducible, es un método útil, económico y fácil de aplicar en las industrias.

### *D. hansenii*: desarrollo de un método rápido de detección (Wrent *et al.*, 2015b)

Con respecto a *D. hansenii*, el desarrollo de metodologías rápidas que permitan la detección diferencial de la especie es muy útil para las industrias. Como ya hemos comentado la identificación de especies a gran escala por análisis de secuencias resulta cara cuando ha de aplicarse a un gran número de cepas y no muy rápida si no se cuenta con esa tecnología en el propio centro. Previamente en nuestro laboratorio habíamos desarrollado un método basado en los perfiles de restricción de la región

IGS del DNA ribosómico que diferencia entre *D. hansenii*, *D. fabryi* y *D. subglobosus* (Quirós *et al.*, 2006). Sin embargo, durante una investigación llevada a cabo en nuestro laboratorio y ajena a esta Tesis, sobre la descarboxilación de sorbato, unos de los conservantes más utilizados en la industria, observamos que *D. hansenii* presentaba una región de 729 pb (LOCUSXM_46154) con una identidad del 69% con la secuencia nucleotídica del gen *PAD1* de *S. cerevisiae* y que a su vez, era muy diferente de la descrita para este gen en otras especies disponibles. Los resultados obtenidos nos hicieron plantear la hipótesis de que podríamos utilizar este gen como diana para el desarrollo de un protocolo de PCR. Hemos diseñado un par de cebadores específicos (DhPADF/DhPADR) que en las condiciones indicadas (Material y Métodos del capítulo 4) amplifican un fragmento muy claro de 400 pb sólo en las cepas de *D. hansenii* ensayadas (100%) pero no en *D. subglobosus* ni en *D. fabryi*, ni en el resto de las otras 22 especies de levaduras analizadas que procedían de diferentes Colecciones de Cultivos Tipo tales como *Torulaspora*, *Hanseniaspora* y *Zygosaccharomyces* y que habitualmente suelen encontrarse en los mismos tipos de alimentos (Tabla 1, capitulo 4). Posteriormente, el protocolo fue validado en cepas aisladas de alimentos (Tabla 2, capitulo 4). Debemos destacar que a diferencia de los cebadores desarrollados por Gente *et al*. (2007), que no reconocen a la cepa de *D. hansenii* CBS 766, cuya posición taxonómica dentro de la especie *D. hansenii* es controvertida, el método que proponemos si lo hace. Al igual que ha ocurrido con algunos de los métodos que hemos desarrollado para otras especies, también nos ha permitido detectar dos cepas cuya identificación, mediante el polimorfismo de los fragmentos de restricción de la región 5,8-ITS del rDNA y el resultado en el medio DDM

(*Debaryomyces* Diferential Medium) (Quirós *et al.*, 2005), era equivocada. En concreto, las cepas de *D. hansenii* EPDI6 y *D. hansenii* Yaa, aisladas en nuestro laboratorio de queso y pionono (un dulce típico) en realidad son cepas de *D. fabryi*. Debemos señalar, que se sabe muy poco de la fisiología y ecología de la especie *D. fabryi*, suele considerarse que proceden de muestras clínicas, aunque el 40% de las cepas se ha aislado de alimentos (Suzuki *et al.*, 2011; Wrent *et al.*, 2014), las cepas EPDI6 e Yaa aumentan este número.

El protocolo que se describe en esta Tesis para la detección de *D. hansenii* (Wrent *et al.*, 2015b) es rápido y asequible. Estos cebadores pueden aplicarse directamente a las colonias con lo que se ahorra mucho tiempo. Sin embargo, recomendamos el método descrito por Lõoke *et al*. (2011) porque el procedimiento dura 15 minutos y el DNA puede almacenarse para futuras amplificaciones. Dado que sólo reconoce las cepas de *D. hansenii* permite diferenciar esta especie de *D. fabryi* o *D. subglobosus*, que son especies indistinguibles fisiológicamente. El protocolo que hemos desarrollado también es útil para la confirmación rápida cuando de todos los aislados a partir de un alimento sólo se busca *D. hansenii* o para la confirmación de esta especie en colecciones privadas o públicas. De hecho muchas cepas que anteriormente se habían clasificado como *D. hansenii* actualmente han sido reclasificadas como de *D. fabryi o D. subglobosus* (Suzuki *et al.*, 2011). Además, consideramos que sería interesante confirmar su utilidad en el ámbito sanitario ya que algunos autores sugieren que *D. hansenii* no sería un patógeno oportunista humano tan frecuente como se piensa (Desnos-Ollivier *et al.*, 2008; Kim *et al.*, 2014) puesto que es habitual que se identifiquen como *C.*

*famata* (anamorfa y coespecífica de *D. hansenii*) cepas que en realidad corresponden a *C. guilliermondii* (anamorfo de *M. guilliermondii*). Los autores lo atribuyen a la identificación mediante sistemas comerciales miniaturizados basados en pruebas morfológicas, como por ejemplo VITECK®2.

***M. guilliermondii*: condicionantes para su crecimiento y capacidad deteriorante en yogures ecológicos. Desarrollo de un método de tipificación intraespecífica (Wrent *et al.*, 2015a; Wrent *et al.*, 2015d)**

El trabajo desarrollado en esta Tesis aborda un caso de deterioro de yogures ecológicos, que como puede verse en el capítulo 6 presentaban una contaminación por esta levadura por encima de $10^6$ UFC/g, analizamos su capacidad para deteriorar el alimento y probamos diferentes métodos de tipificación de cepas con objeto de seleccionar el más idóneo.

El interés de los consumidores por productos más "naturales" hace que haya aumentado la demanda de alimentos ecológicos (*organics* en inglés). Los yogures analizados en este estudio se obtenían por fermentación de leche ecológica pasteurizada sin la adición posterior de aditivos artificiales. Ya predijo Stratford en 2006 que la disminución en la utilización de los conservantes aumentaría el número de alimentos deteriorados. El estudio lo realizamos con muestras de yogures ecológicos enviados por el laboratorio de control de calidad de la industria a lo largo de dos años. Aunque algunas levaduras son importantes para la maduración de los quesos o para la producción de alimentos fermentados típicos del este de Europa o Asia, como por ejemplo el *Kefir,* en la producción de yogur las buenas prácticas de fabricación indican que los yogures deben contener menos de 1 levadura por gramo (Davis, 1975). La

especie *M. guilliermondii* no se encuentra entre las levaduras que aparecen normalmente en los yogures, para una revisión ver (Deák, 2008; Viljoen *et al.*, 2003) y en nuestro conocimiento no se han publicado previamente problemas de deterioro de yogures causados por esta levadura. La Tabla 1, capítulo 6 muestra los yogures analizados, el deterioro que presentaban y los recuentos de levaduras que se obtuvieron en ellos. Como puede observarse el deterioro sólo se produce, como cabía esperar, en los yogures que contenían frutas. Es habitual aislar cepas de *M. guilliermondii* de frutas (Deák, 2008), por lo que es lógico que la adición de ésta a los yogures proporcione una fuente de azúcar fermentable que propicie el deterioro por producción de gas. Sin embargo, nos sorprendió que también los yogures naturales presentaran una importante contaminación por levaduras. Esto nos animó a analizar los factores que permitían su desarrollo en los mismos. Está extendida la idea de que el deterioro de yogures se debe a un pequeño grupo de levaduras que fermentan lactosa (Fleet, 1990), cuando *M. guilliermondii* no lo hace y, además, fermenta débilmente otros azúcares (Kurtzman y Suzuki, 2010). Cuando re-inoculamos esta levadura nuevamente en yogures (Tabla 3, capítulo 6) pudimos comprobar que se reproducía el crecimiento y el deterioro en los yogures de fruta y el abundante crecimiento en los yogures naturales. Aunque se trata de una levadura poco fermentativa si comparamos con *S. cerevisiae*, su crecimiento es tan denso que es suficiente para hinchar los envases (Tabla 3, capítulo 6) y Fig. 7.1.


**Figura 7.1.** La figura muestra dos yogures, un yogurt con frutas (7) y otro natural (8). El yogurt con fruta aparece hinchado (7).

Por otro lado, parece que se establece una relación sinérgica entre las bacterias lácticas (LABs) y las levaduras, con las LABs produciendo fuentes de carbono a partir de la lactosa y las levaduras evitando el efecto negativo de la bajada del pH en las primeras al consumir el ácido láctico (Viljoen *et al.*, 2003). La cepa estudiada es capaz de crecer en presencia de lactato y de lactato más galactosa. Como puede verse en la (Tabla 3, capítulo 6) los estudios realizados a 8ºC refuerzan la importancia de mantener la cadena del frío para evitar el deterioro, no así el crecimiento que sólo se retrasa. Esto puede explicarse si consideramos que la fermentación del azúcar no sería la fuente principal de obtención de ATP. *M. guilliermondii* es capaz de oxidar lactato y consecuentemente puede crecer sin fermentar. Nuestra hipótesis es que el crecimiento oxidativo sería previo al fermentativo que sólo se produciría cuando el oxígeno se agotara. El mantenimiento a 8ºC no produciría el rechazo del consumidor

(no se detecta la contaminación) pero, en nuestra opinión, habría que analizar si la ingesta de una concentración tan elevada de células de *M. guilliermondii* produce algún efecto negativo en personas con un sistema inmune débil. Por otro lado, las levaduras no suelen deteriorar los yogures que incluyen ácido sórbico o sus sales, en esas condiciones las levaduras alcanzarían $10^4$ UFC/g después de dos meses a 20ºC (Mataragas *et al.*, 2011), una concentración muy inferior a la alcanzada en los yogures que hemos estudiado en este trabajo y que no incorporaban estos agentes conservantes.

Se han descrito muy pocos métodos para la discriminación de cepas en *M. guilliermondii*. Algunos autores consideran, además, muy necesario la diferenciación entre cepas con potencial patogénico (Corte *et al.*, 2015; Romi *et al.*, 2014) y proponen la necesidad de estudios de tipificación de cepas. Martorell *et al*. (2006) utilizaron la técnica de RFLP del DNA mitocondrial para diferenciar cepas que produjesen el compuesto 4-ethylphenol, que confiere un olor desagradable al vino. Sin embargo, Romi *et al*. (2014) comprobaron que esta técnica en realidad no permite diferenciar cepas de *M. guilliermondii* sino que diferencia entre dos especies *M. guilliermondii* y *M. caribbica*. Lopes *et al*.(2009), utilizaron la combinación de la amplificación al azar de fragmentos de DNA polimórfico (RAPD) y la producción de toxina *killer* para detectar el potencial deteriorante de las cepas de esta especie en vinos. No obstante, algunos autores consideran que la técnica de RAPD produce productos de amplificación que son artefactos (Albertin *et al.*, 2014). Por ello, con el objetivo de obtener un método adecuado para la tipificación de cepas de *M. guilliermondii* en esta Tesis (Wrent *et al*., 2015d) se han comparado

tres métodos: El perfil de restricción del DNA mitocondrial previamente desarrollado por Martorell *et al*. (2006), el perfil de restricción del fragmento amplificado de la región IGS (IGS-PCR RFLP) previamente desarrollado en nuestro laboratorio para otras levaduras (Quirós *et al.*, 2006; Romero *et al.*, 2005; Wrent *et al.*, 2010) y el uso de un método basado en la detección de microsatélites (Wrent *et al.*, 2015d).

Nuestros resultados coinciden con los obtenidos por Martorell *et al*. (2006) en el sentido de que los perfiles obtenidos con la endonucleasa *Hinf*I (RFLP-mtDNA) proporcionan variabilidad entre las especies y está variabilidad está posiblemente relacionada con características fisiológicas. Por ejemplo, todas las cepas aisladas de los yogures presentan el patrón A al igual que la cepa de *M. guilliermondii* CECT 1438 que procede de pozol, una bebida típica mejicana que se obtiene por fermentación láctica del maíz (Tabla 1, capítulo 6). Por lo tanto, consideramos que el patrón obtenido correspondería a las cepas lactato positivas. Igualmente, hemos podido comprobar que el patrón D corresponde a dos cepas aisladas de diferentes lugares pero ambas de suelo, por lo que suponemos que corresponde a cepas con alguna actividad enzimática concreta de utilidad en ese ambiente. Con respecto a la técnica IGS-PCR RFLP hemos podido comprobar que, con las enzimas ensayadas (*Hae*III, *Hap*II, *Hha*I y *Mbo*I), produce una menor discriminación entre cepas que el método RFLP-mtDNA. Sin embargo, una combinación de ambos métodos diferencia cada una de las cepas de colecciones de Cultivo Tipo incluidas en este estudio entre ellas y éstas con las aisladas de los yogures ecológicos, que presentan un único e idéntico patrón (Tabla 1, capítulo 6). Los resultados parecían indicar que todas las cepas aisladas de la fábrica de yogures

serían la misma. Sin embargo, comparando estos resultados con los obtenidos mediante la técnica de detección de microsatélites, los resultados varían como comentaremos a continuación.

El genotipado con microsatélites se ha utilizado mucho para estudios evolutivos, de poblaciones y ecológicos en diferentes especies (Rosenberg *et al.*, 2002; Schlötterer, 2001) y para el trazado de productos cárnicos y lácticos, con objeto de certificar la identidad y el origen de los productos (Sardina *et al.*, 2015; Shackell *et al.*, 2005). Como hemos comentado en el capítulo 1, se ha aplicado para la tipificación intraespecífica de las especies de levadura: *B. bruxellensis*, *C. albicans*, *S. cerevisiae* y *S. uvarum* (Albertin *et al.*, 2014; Antonangelo *et al.*, 2013; Hennequin *et al.*, 2001; L'Ollivier *et al.*, 2012; Zhang *et al.*, 2015) comprobándose que se trata de un método que revela muy eficientemente el polimorfismo entre cepas. Los microsatélites, como hemos comentado en el capítulo de Introducción son grupos de nucleótidos repetidos (1 a 6) que están presentes en todos los genomas eucariotas. Este trabajo analiza por primera vez los microsatélites presentes en *M. guilliermondii*. De todos los posibles, hemos seleccionado aquellos que aparecen con más frecuencia en levaduras (repeticiones de dos, tres, y cuatro nucleótidos) (Lim *et al.*, 2004). Para ello, fue fundamental contar con los nueve fragmentos secuenciados que están publicados en las bases de datos y la utilización del *Software Tandem Repeat Finder*. Como puede verse en la Tabla 2, capítulo 5 en dos de los fragmentos analizados no se encontraron microsatélites. Cuestión que ha sido abordada previamente por otros autores y que ha llevado a la conclusión de que los microsatélites son menos frecuentes o inexistentes

en los genomas fúngicos que en otros taxones (Tóth *et al.*, 2000). En el resto de fragmentos se han podido detectar un total de diecinueve microsatélites (Tabla 2, capítulo 5), para los que se han diseñado cebadores específicos (Tabla 2, capítulo 5). De los cuatro pares de cebadores probados, sc15F/R, sc22F/R sc32F/R y sc72F/R resultaron ser específicos para *M. guilliermondii* dos de ellos (sc15F/R, y sc72F/R). Sin embargo, el análisis (ver capítulo 5, sección Material y Métodos) de la combinación de los fragmentos obtenidos con los cebadores amplificados sc15F/R, sc32F/R y sc72F/R permitió diferenciar en una sola etapa todas las cepas de colección utilizadas. Además, los resultados pudieron ser validados cuando se aplicaron tanto a las cepas aisladas de los yogures como a otras de diferentes nichos ecológicos. Es destacable que una especie como *D. hansenii*, que como hemos comentado es frecuentemente identificada como *M. guilliermondii* presenta resultados negativos con esta técnica, al igual que el resto de especies aisladas de alimentos elegidas para comparación (Tabla 1, capítulo 5). Mientras que otras especies indistinguibles fisiológicamente de *M. guilliermondii* como *M. caribbica* o *C. carpophyla* (perteneciente también al *cluster* de *Meyerozyma*) dan lugar a fragmentos amplificados sólo con los cebadores sc32F/R y sc22F/R y sc32F/R respectivamente. Es de destacar, que en el caso de *M. caribbica* la secuenciación de la región D1/D2 del rDNA no aporta una identificación clara (Romi *et al.*, 2014), por lo que pensamos que la utilización de la técnica que proponemos puede ayudar a diferenciar esta especie de *M. guilliermondii* y sería interesante, valorar su utilidad para la tipificación de cepas de esta especie. Es más, La combinación de los pares de cebadores que proponemos nos ha permitido detectar cepas de *M. caribbica* identificadas como *M. guilliermondii* (CECT

10100, ISA 2105, ISA 2375 e ISA 2376). Por otro lado, dentro del *cluster* de *Meyerozyma* se clasifican además de las especies ya mencionadas, *M. caribbica* y *C. carpohila*, otras especies todas ellas aisladas de insectos y, de las que en general sólo se ha aislado una o dos cepas *C. athensensis*, *C. elateridarum*, *C. smithsonii* (Kurtzman, 2011a). Hemos podido comprobar que aunque pueden obtenerse fragmentos amplificados con algunos de los cebadores dan lugar a un conjunto de bandas que no interfieren con el resultado obtenido para *M. guilliermondii*.

De acuerdo con las ventajas descritas por Albertin *et al*. (2014) para *B. bruxelensis*, con respecto a la reproducibilidad entre laboratorios y al gran polimorfismo obtenido, hemos podido comprobar que la selección de cebadores que utilizamos amplifica fragmentos que son estables a lo largo del tiempo, pues se han analizado dos cepa (CECT 1456$^{T}$ (CBS 2030$^{T}$) y CECT 1438 (CBS 6557), mantenidas durante años en otro laboratorio además del nuestro, obteniéndose idéntico resultado para cada una de ellas. Como cabía esperar, el polimorfismo fue menor dentro de cada nicho ecológico ya que la misma cepa puede aislarse de varias muestras pero no se ha dado el caso de que cepas de diferentes nichos ecológicos presenten el mismo patrón. En la Tabla 1, (capítulo 5) se muestran los resultados. Aquí nos centraremos en el caso de las cepas aisladas de los yogures ecológicos. Como puede observarse las tres cepas aisladas de las mermeladas de fruta (M1, M2 y Ma11.1) son la misma y aparecen en el yogur de albaricoque un año después. Estos resultados parecen indicar que el tratamiento térmico al que se somete a la fruta en su elaboración no afectaría significativamente a esta levadura. Esto puede explicarse por el efecto protector que ejerce el azúcar (Marquina *et al.*, 2001). Sin

embargo, a diferencia de los resultados obtenidos con la combinación de las técnicas RFLP-mtDNA y IGS-PCR RFLP está no sería la única cepa de *M. guilliermondii* que ha contaminado la fábrica.

Destacamos, asimismo, que este método nos ha permitido discriminar entre cepas, CBS 6557 (CECT 1438) y CBS 6021 (CECT 1021), que no diferencian otros métodos como i-SSR $(GACA)_4$ (Corte *et al.*, 2015). Algunas de ellas han sido también utilizadas, por otros autores, en estudios de tipificación de cepas productoras de 4-etilfenol en vinos. Los resultados de esa tipificación fue utilizada por los autores para diferenciar las cepas poco productoras de las muy productoras (Martorell *et al.*, 2006). Teniendo en cuenta que valores de 620 µg/L dan lugar a un olor desagradable (Chatonnet *et al.*, 1992) y por el contrario, niveles inferiores a 400 µg/L producen el efecto contrario (Loureiro y Malfeito-Ferreira, 2003), la cuestión de si las cepas son poco productoras o muy productoras es realmente importante. En este trabajo hemos podido comprobar que las cepas catalogadas por estos autores como poco productoras (Martorell *et al.*, 2006) y que también hemos analizado nosotros (ISA 2110 e ISA 2125), son *M. guilliermondii*. Mientras que dentro de las muy productoras se incluye una de las identificadas en este trabajo (ISA 2105) como *M. caribbica*. Consideramos que sería interesante clarificar este punto porque quizás las propiedades como deteriorantes de vinos atribuidas a *M. guilliermondii* correspondan a *M. caribbica*.

# Capítulo 8

**Conclusiones**

1. El análisis de los fragmentos de restricción de la región IGS del DNA ribosómico diseñado en esta Tesis constituye un método de tipificación de cepas discriminatorio y reproducible para las especies *Z. bailii*, *Z. mellis* y *Z. rouxii* y es fácil de implantar en la rutina de un laboratorio industrial.
2. *Z. rouxii* CECT 11923 y CECT 10425 son cepas híbridas posiblemente entre las especies *Z. rouxii* y *Z. sapae,* y *Z. pseudorouxii* y *Z.sapae* respectivamente.
3. Existen diferencias en la secuencia de la región IGS1 del DNA ribosómico entre las cepas híbridas y las que no lo son. Los cebadores específicos (HibZR/HibZF) que reconocen esta región, constituyen un método rápido y económico para la detección de cepas híbridas dentro del género *Zygosaccharomyces*.
4. Las cepas de la especie *D. hansenii* pueden ser identificadas rápida y económicamente a partir de diferentes productos alimenticios con los cebadores específicos (DhPADF/DhPADR). Éstos permiten diferenciar esta especie de *D. fabryi* o *D. subglobosus*, que son indistinguibles fisiológicamente. El método puede aplicarse directamente sobre las colonias, aunque recomendamos el método de Lõoke *et al*., (2011) y en quince minutos el DNA extraído puede amplificarse mediante PCR.
5. La detección de microsatélites constituye el mejor método de los ensayados en este trabajo y hasta este momento para la discriminación de cepas de *M. guilliermondii*. El análisis combinado de los resultados obtenidos con los tres cebadores (sc15F/R, sc32F/R y sc72F/R) reduce el tiempo y el coste. Además, permite

distinguir esta especie de otras fisiológicamente idénticas como *M. caribbica o C. carpophila*.

6. *M. guilliermondii* es la levadura responsable del deterioro de los yogures ecológicos. El gas producido se debe a la fermentación del azúcar de las mermeladas añadidas. La capacidad de crecer en lactato como fuente de carbono permite que esta especie se multiplique también en los yogures naturales hasta altas concentraciones $10^6$ UFC/g sin que el consumidor lo perciba. A 8ºC continúa el crecimiento aunque la fermentación se inhibe casi totalmente. Pensando en los consumidores inmunodeprimidos debería considerarse el control de la especie *M. guilliermondii* por razones tanto de calidad como sanitarias. La aplicación de la tipificación de cepas mediante la detección de microsatélites indica que la empresa se contaminó al menos por dos cepas, una de ellas procedente de las mermeladas de fruta.

# Capítulo 9

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| **AFPL** | Polimorfismo en la longitud de los fragmentos amplificados |
| **ATCC** | Colección Americana de Cultivo Tipo |
| **$a_w$** | Actividad de agua |
| **CBS** | *Centraalbureau voor Schimmelcultures*(Colección de cultivos fúngicos) |
| **CECT** | Coleccion Española de Cultivo Tipo |
| **clado CTG** | Especies de levaduras que traduce CTG a serina en vez leucina |
| **Cluster** | Grupo |
| **CoQ-9** | Coenzima Q-9 |
| **D1/D2** | Dominio D1 y D2 del gen 26S r DNA |
| **DGGE** | Electroforesis en gel desnaturalizante en gradiente |
| **DNA** | Ácido desoxirribonucleico |
| **EMBL** | Laboratorio Europeo de Biología Molecular |
| **FTIR** | Espectroscopia de Infrarrojos por Transformada de Fourier |
| **IGS** | Espaciador Intergénico del rDNA |
| **IMF** | *Intermediate Moisture Food* (alimentos con actividad de agua intermedia) |
| **ITS** | Espaciador Intergénico tránscrito del rDNA |
| **LSU** | Subunidad grande del rDNA |
| **M** | Molar |
| **MALDI-TOF MS** | Espectrometría de masas de ionización mediante láser asistida por una matriz |
| **Mb** | Megabyte |
| **mtDNA** | DNA mitocondrial |
| **MUCL** | Mycotheque de l'Universite Catholique de Louvain (Micoteca de la Universidad Católica de Lovaina) |
| **NCYC** | *National Collection of Yeast Cultures, UK* (Colección nacional de levaduras, Reino Unido) |
| **pb** | Pares de Bases |
| **PCR** | Reacción en Cadena de la Polimerasa |
| **RAPD** | Amplificación aleatoria de DNA polimórfico |
| **rDNA** | DNA ribosómico |
| **RFLP** | Polimorfismo en la longitud de los fragmentos de restricción |
| **rRNA** | RNA ribosómico |
| **SSU** | Subunidad pequeña del rDNA |
| **$T_m$** | Temperatura de Fusión |
| **UFC/g** | Unidad formadora de colonia/gramo |
| **YMA** | *Yeast Morphology Agar* |
| **YMB** | *Yeast Morphology Broth* |
| **26S** | Gen 26S del rDNA |
| **5,8S** | Gen 5.8S del rDNA |

Las abreviaturas que no se indican siguen el código de la IUPAC